Evidence for gene transfer and expression of factor IX in haemophilia B patients treated with an AAV vector

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Pre-clinical studies in mice and haemophilic dogs have shown that introduction of an adeno-associated viral (AAV) vector encoding blood coagulation factor IX (F.IX) into skeletal muscle results in sustained expression of F.IX at levels sufficient to correct the haemophilic phenotype[1,2]. On the basis of these data and additional pre-clinical studies demonstrating an absence of vector-related toxicity, we initiated a clinical study of intramuscular injection of an AAV vector expressing human F.IX in adults with severe haemophilia B. The study has a dose-escalation design, and all patients have now been enrolled in the initial dose cohort (2×10^{11} vg/kg). Assessment in the first three patients of safety and gene transfer and expression show no evidence of germline transmission of vector sequences or formation of inhibitory antibodies against F.IX. We found that the vector sequences are present in muscle by PCR and Southern-blot analyses of muscle biopsies and we demonstrated expression of F.IX by immunohistochemistry. We observed modest changes in clinical endpoints including circulating levels of F.IX and frequency of F.IX protein infusion. The evidence of gene expression at low doses of vector suggests that dose calculations based on animal data may have overestimated the amount of vector required to achieve therapeutic levels in humans, and that the approach offers the possibility of converting severe haemophilia B to a milder form of the disease.

Haemophilia B is the bleeding diathesis resulting from mutations in the gene encoding F.IX (*F9*), a proenzyme required for generation of a fibrin clot. The clinical severity of haemophilia B correlates closely with circulating levels of F.IX: individuals with less than 1% of normal activity are severely affected, whereas those with levels 1–5% of normal generally have a more moderate course. Current treatment is based on intravenous infusion of clotting factor concentrates; regimens in which factor is infused prophylactically, with a goal of maintaining factor levels greater than 1% at all times, have been shown to prevent most of the joint damage and life-threatening bleeding complications of the disease[3,4]. Thus, the goal of gene therapy for haemophilia B is the sustained expression of F.IX at levels more than 1% of normal. This goal has been achieved in mice and haemophilic dogs by introducing an AAV vector expressing F.IX into skeletal muscle. Intramuscular injection of an AAV vector expressing human F.IX into immunodeficient mice caused expression of F.IX at 5–7% of normal human plasma levels for more than 12 months (vector dose of 1×10^{13} vector genomes (vg)/kg; ref. 1). Subsequently, intramuscular injection of an AAV vector expressing canine F.IX in dogs with haemophilia B resulted in expression of F.IX at levels up to 1.4% of normal (vector dose of 8.5×10^{12} vg/kg; ref. 2). The levels of expression in these haemophilic dogs are currently stable more than 2.5

Table 1 • Clinical data for patients A, B and C

	A	B	C
Age	38	23	67
Race	European	Asian	European
Baseline F.IX activity level	<1%	<1%	<1%
Baseline F.IX antigen level	24%	<1%	<1%
Mutation	Arg-4→Leu nt 6,365 CGG→CTG	Ala 351→Pro nt 31,172 GCT→CCT	Gly 114→Arg nt 17,755 GGA→CGA
Viral status			
HIV	positive	negative	negative
hepatitis C	positive	positive	negative
hepatitis B	negative	negative	positive
hepatitis A	negative	negative	negative
Significant medical history	s/p GI bleed; s/p seizures secondary to bilateral epidural haematomas; s/p eosiniphilic granuloma R parietal skull; s/p knee synovectomy and arthroscopy	s/p GI bleed; s/p nephrectomy secondary to iliopsoas bleed	s/p GI bleed; adult onset diabetes mellitus
Current medications	ritonavir, lamivudine, stavudine, oxycodone prn	oxycodone prn	glyburide

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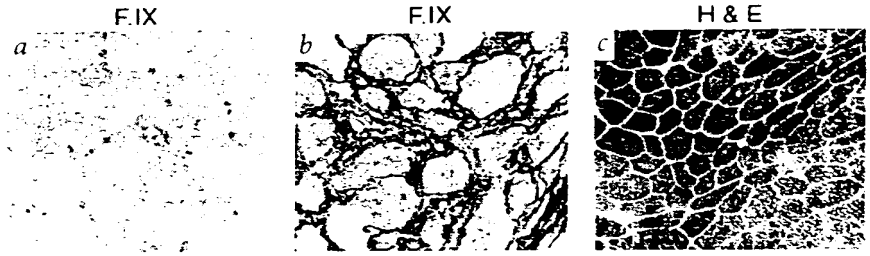


Fig. 1 Histochemical analysis of muscle biopsy. Immunoperoxidase staining of F.IX is shown for cross-sections of muscle tissue of a negative control (*a*) and a vector-injected patient (*b*). The dark brown staining for F.IX is seen in the extracellular matrix surrounding muscle fibres. Original magnification ×200. *c*, Haematoxylin-and-eosin-stained cross-section of muscle tissue from a patient injected with vector. Original magnification ×100. Muscle biopsies were performed 2–3 months after vector administration.

years after the initial and only injection (R.W.H., K.A.H. and T. Nichols, unpublished data).

Clinical data on our first three patients are shown (Table 1). Evidence for gene transfer and expression following vector administration was sought directly by muscle biopsy and indirectly by measurement of circulating F.IX levels and assessment of bleeding episodes and frequency of clotting factor infusion. We performed muscle biopsies 8–12 weeks after vector administration; PCR on DNA extracted from injected muscle was positive for vector sequences in all three patients (data not shown). Immunohistochemical staining of skeletal muscle was positive for F.IX in the extracellular space, a pattern that had been documented in pre-clinical studies for F.IX secreted by muscle fibres[1] (Fig. 1*a*,*b*). Additional sections analysed by routine histology showed no evidence of inflammation or muscle injury (Fig. 1*c*, and data not shown). Results of coagulation assays and records of factor usage for patients A and B are shown (Fig. 2 and Table 2). Patient A, who was documented to have a baseline F.IX level of less than 1% by three clinical coagulation laboratories, demonstrated a level of more than 1% (also documented by three clinical coagulation laboratories) on multiple occasions beginning approximately 8 weeks after vector administration. These levels were drawn at time points at least 14 days after the most recent factor infusion, eliminating the possibility that the levels reflected a contribution from residual infused factor. Patient B showed a small change in F.IX level, remaining less than 1% of normal (Table 2), but both patients showed a reduction in clotting factor consumption following treatment with the AAV vector (Fig. 2). The treatment time lines are given in 20-day intervals; the first half of the time line (pre-AAV treatment) serves as a control for the second half (post-AAV treatment). Patient A has experienced a 50% reduction in factor usage sustained over a period of more than 100 days, and patient B has experienced an 80% reduction in factor usage also sustained over a period of more than 100 days. Patient C, despite a F.IX level of less than 1%, treats himself infrequently (so-called 'mild-severe'[5]), typically less than four times per year. Since vector injection five months ago, he has had no change in clinical status or F.IX levels (data not shown). Gene transfer and expression, however, were documented by Southern blot on DNA extracted from a muscle biopsy specimen, which showed approximately one vector genome copy per diploid genome, and by RT–PCR, which was positive for F.IX expression (data not shown).

Major safety issues to be addressed here include the risk of formation of inhibitory antibodies to the transgene product, which can block treatment by conventional protein therapy, and the risk of inadvertent germline transmission of vector sequences. Evidence for formation of anti-F.IX antibodies was sought by two different methods, the standard Bethesda assay and a western-blot method. Bethesda assay performed weekly through the first eight weeks, then biweekly through the next four months, showed no evidence of inhibitor formation (data not shown). Western-blot analysis, which detects both inhibitory and non-inhibitory antibodies, has also been consistently negative for evidence of antibodies (Fig. 3*a*). The clinical responses of the patients to infused factor, and a pharmacokinetic study completed in one patient (data not shown), support these laboratory studies, because all patients continue to exhibit excellent responses to clotting factor concentrates.

Pre-clinical biodistribution studies in mice and rabbits carried out at doses 50-fold higher than those used here demonstrated that AAV vector introduced into sites in skeletal muscle remains largely confined to that tissue. Specifically, there is no evidence of distribution of vector into the semen (ORDA web site, http://www.nih.gov/od/orda/3-99RAC.htm) despite transient low-level positive signals in serum 24 hours after injection.

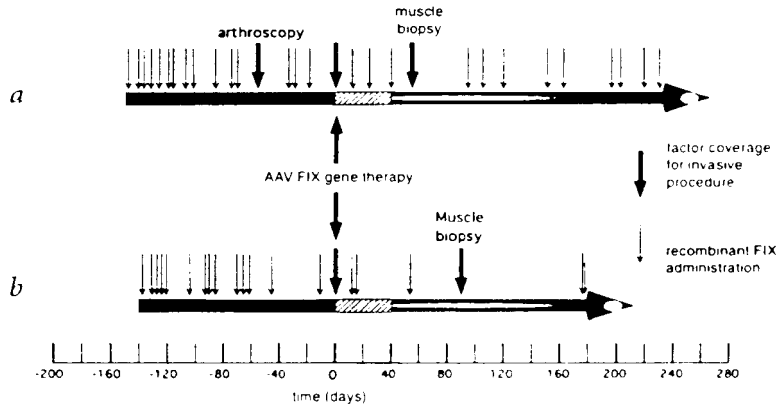


Fig. 2 Factor usage for patients A and B. The horizontal line denotes time; the scale at the bottom is marked in 20-day increments. Arrows denote infusion of F.IX concentrate for spontaneous bleeds (thin arrows) or invasive procedures (thick arrows). The thick vertical arrow in the middle of the chart denotes the date of vector infusion. The hatched bar on the timeline denotes the initial six-week period during which transgene expression is expected to be low based on animal studies[2]. All patients have baseline F.IX levels <1%. *a*, For patient A, F.IX was documented to be 1%, with an activated partial thromboplastin time (aPTT) of 61 s, when he presented for muscle biopsy at 8 weeks following injection. On the day of and 1 d after muscle biopsy, the patient received F.IX concentrate, after 17 d, with no intervening factor treatment, the F.IX level was 1.6% with an aPTT of 48 s. Ten days later, the F.IX level was determined to be 1.4% with an aPTT of 47 s, again with no intervening factor treatment. Ten days later the patient treated himself with concentrate for atypical knee pain, and a F.IX level drawn after 4 d was 3.7% with an aPTT of 41 s, reflecting the recent protein infusion. A blood sample drawn 14 d after a subsequent treatment showed a F.IX level of 1.3% with an aPTT of 50 s. Over the ensuing weeks the factor level was measured in the 0.5–1.0% range, with aPTTs in the range of 50 s. Factor infusion is reduced ~50% from baseline. *b*, The baseline F.IX level of patient B is <0.3%, his baseline factor infusion is ~2–5 times/month. Despite no substantial change in F.IX level, patient B's factor consumption has decreased by >80%.

Table 2 • Coagulation data[a] for patients A and B

	Patient A[b]			Patient B[b,c]		
	F.IX	aPTT		F.IX	aPTT	
Baseline	<1%			<0.3%		
Week 6	<0.3%	82.9				
Week 8	1%	61		<0.3%	102	
Week 10	1.6%	48		0.3%	91.2	
Week 12	1.4%	46.8		0.3%	102.3	
Week 14	3.7%	41.0	(post-F.IX infusion)	3.0%	52.6	(post-F.IX infusion)
Week 17	1.3%	50.6		0.4%	72	
Week 18	0.8%	49.4				
Week 20	0.5%	54.1		0.4%	107	
Week 22	0.9%	53.7				
Week 24	0.5%	52.1		0.8%	65.5	

[a]Unless otherwise noted, all data points were drawn at least 14 d after the most recent factor transfusion. [b]Data generated in CHOP Clinical Coagulation Laboratory. [c]Data generated in Stanford University Clinical Coagulation Laboratory.

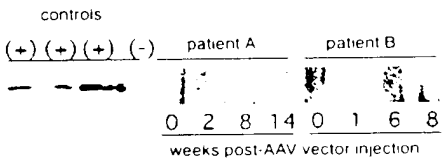
PCR analysis for vector sequences in body fluids from patients (data not shown) is in agreement with the pre-clinical studies, as serum samples were positive for vector sequences at 24 and 48 hours after injection, but were negative thereafter. Saliva samples were also positive at 24 hours after injection, but were subsequently negative, and one patient had a positive urine sample at 24 hours with all subsequent urine samples being negative. All remaining samples, including serial semen samples collected out to 48 days, 56 days and 59 days after injection, were negative for vector sequences.

The effect on transduction efficiency in skeletal muscle of neutralizing antibodies against AAV serotype 2 is unknown[6]. All patients enrolled in this study had detectable titres of neutralizing antibodies against AAV before treatment, with the titre varying over a range of two logs, from 1:10 to 1:1,000 (Fig. 3*b*). The rise in neutralizing antibody titre following vector administration varied from 10- to 1,000-fold. The highest pre-treatment antibody titre was in patient B, whose post-injection muscle biopsy is positive for F.IX expression by immunohistochemical staining (Fig. 1*b*), arguing against any inhibitory effect of the antibodies on skeletal muscle transduction. Additional laboratory studies, including serial complete blood counts and serum chemistries, disclosed no treatment-related abnormalities (data not shown).

Despite promising pre-clinical data, clinical experience with AAV vectors is limited; our study is the first in which AAV vectors have been introduced into skeletal muscle. On the basis of these initial patients, the approach appears to be safe, with no evidence at this dose for toxicity related to vector administration, inadvertent germline transmission of vector sequences or formation of inhibitory antibodies to the transgene product. Moreover, biopsy of injected sites shows evidence of gene expression by immunofluorescence staining. Notably, one of the patients in the initial low-dose cohort showed detectable circulating levels of F.IX above 1%. On the basis of studies in mice and haemophilic dogs[1,2], we had predicted that the patients in the low-dose group would not show measurable levels of F.IX expression (Table 3). Our observations suggest that the vector may be more efficient in humans than in mice or dogs; indeed, we have observed this to be the case in tissue culture, where we have measured as much as a 2-log difference in copy number of the donated gene in primary cultures of human versus mouse muscle cells (unpublished data). Because the vector is engineered from a virus that infects humans but not rodents, the processes of vector binding and entry[7,8] may be more efficient in human cells than in those of other species. A similar consideration applies to the CMV promoter-enhancer used in the vector; because CMV infects humans but not other species, the promoter may have evolved to express most efficiently in the setting of human transcription factors. An objective of dose escalation will be to identify a dose at which all patients express F.IX levels of more than 1%.

The fact that F.IX levels of just above 1% in patient A were associated with a reduction in factor use is consistent with the findings of the Swedish prophylaxis studies, which showed a reduction in haemorrhages when concentrate was dosed to maintain nadir levels of approximately 1% (refs 3,4). The reduction in bleeding seen in patient B raises the question of whether levels of F.IX less than 1% can also result in a reduction in clinical bleeding episodes. More data will be required to resolve this issue. The difference in factor levels seen among patients A, B and C may be accounted for by biologic variation, but another factor that may be important is the presence or absence of circulating F.IX antigen (Table 1). The volume of distribution of F.IX includes both the intravascular and extravascular space, where F.IX can bind tightly to collagen IV (ref. 9). Saturation of these potential binding sites in individuals with circulating F.IX protein may result in higher levels of the donated gene product in the

a



b

time post vector injection	patient A	patient B	patient C
pre-treatment	1:100	1:1,000	1:10
1 week	1:1,000	1:10,000	1:1,000
2 weeks	1:5,000	1:10,000	1:10,000
4 weeks	NS	1:10,000	1:10,000
6 weeks	NS	1:10,000	NS
8 weeks	1:5,000	1:10,000	NS
12 weeks	1:1,000	NS	NS

Fig. 3 Immune responses to AAV-CMV-hF.IX. ***a***, Western-blot analysis of anti-human F.IX in serum samples of haemophilia B patients. Plasma-derived human F.IX is transferred to a membrane, which is incubated with serum samples from patients. Lanes 1,2, positive control (+) (patient with inhibitory anti-F.IX) diluted 1:2,000; lane 3, positive control (+) diluted 1:1,000; lane 4, negative control (–); lanes 5–8, samples from patient A pretreatment (0 weeks, lane 5) and 2 weeks (lane 6), 8 weeks (lane 7) and 14 weeks (lane 8) following AAV-vector injection; lanes 9–12, samples from patient B pretreatment (0 weeks, lane 9) and 1 week (lane 10), 6 weeks (lane 11) and 8 weeks (lane 12) post-injection. ***b***, Neutralizing antibody titres against AAV before and after treatment with AAV-CMV-hF.IX. NS, no sample available at time of assay.

Table 3 • Predicted levels of circulating F.IX in humans

Dose	F.IX level in mice[a]	F.IX level in dogs[b]	Predicted level in humans[c]	Predicted % normal levels in humans
2×10^{11} vg/kg	6 ng/ml	2–4 ng/ml	2–6 ng/ml	≤0.1%
2×10^{12} vg/kg	60 ng/ml	16 ng/ml	16–60 ng/ml	0.3–1.2%
1×10^{13} vg/kg	300 ng/ml	80 ng/ml	80–300 ng/ml	1.8–6%

[a]Predicted plasma F.IX level in mice based on mouse experimental data[1]. [b]Predicted plasma F.IX level in dogs based on canine experimental data[2]. [c]Extrapolated from studies in animals.

circulation. The data gathered so far indicate that AAV-mediated gene therapy for haemophilia B is safe and has the potential to demonstrate efficacy, although testing at higher doses will be required to confirm this interpretation. This treatment strategy thus offers the possibility of converting severe haemophilia B to a milder form of the disease through a relatively non-invasive procedure. In the broader context of gene-based treatment of inherited diseases, the record so far has been discouraging, with no clear-cut evidence of success with *in vivo* gene therapy. Our results indicate that *in vivo* administration of viral vectors offers the possibility of improving the clinical course of genetic diseases that affect many individuals worldwide.

Methods

Clinical protocol. Our study was designed as an open-label, dose-escalation Phase I trial. The clinical protocol was reviewed and approved by the Institutional Review Boards of The Children's Hospital of Philadelphia (CHOP), Stanford University and the University of Pittsburgh Medical Center, the Institutional Biosafety Committees at CHOP and Stanford (the institutions where vector is injected), the Center for Biologics Evaluation and Research of the U.S. Food and Drug Administration, and the NIH Office for Recombinant DNA Activities. Inclusion criteria for the study include severe haemophilia B with F.IX level ≤1%, life expectancy of at least one year, male sex, age ≥18 years, >20 days exposure history to F.IX concentrates and ability to give informed consent. Exclusion criteria include acute infectious illness, end-stage renal disease, severe liver disease defined as bilirubin >2 times normal, transaminases >5 times normal or alkaline phosphatase >5 times normal, platelet count <50,000, presence of inflammatory muscle disease, unwillingness to practice birth control until three semen samples are documented to be negative for vector sequences and unwillingness to stop a regimen of prophylactic clotting factor infusion. The mutation in *F9* was determined for each patient by the dideoxynucleotide chain termination method following PCR amplification of the eight exons of *F9* from genomic DNA that had been isolated from patient blood samples.

Preparation of AAV-CMV-hF.IX. Vector was prepared in a GMP facility (Avigen) using a triple-transfection procedure[10,11]. The F.IX expression plasmid is an 11,442-bp plasmid containing the cytomegalovirus (CMV) immediate early promoter, exon 1 of *F9* (ref. 12), a 1.4-kb fragment of *F9* intron 1 (ref. 13), exons 2–8 of *F9*, 227 bp of *F9* 3′ UTR and the SV40 late polyadenylation sequence between two AAV inverted terminal repeats. The rep/cap plasmid pHLP19 and the helper adenovirus plasmid pLadeno5 have been described[10,11]. Recombinant AAV was produced by transfecting the three plasmids into HEK 293 cells by calcium phosphate transfection. Following incubation to allow vector amplification, cells were lysed and treated with nuclease to reduce residual cellular and plasmid DNA. After precipitation, vector was purified by two cycles of isopycnic ultracentrifugation; fractions containing vector were pooled, dialysed and concentrated. The concentrated vector was formulated, sterile filtered (0.22 μM) and aseptically filled into glass vials. Vector genomes were titred by a quantitative dot-blot assay in which the signal from aliquots of test material is compared with a standard curve generated using the linearized F.IX expression plasmid. The vector underwent in-process and final product testing as described[14].

Vector administration. After giving informed consent, patients were admitted to the Clinical Research Center at either CHOP or Stanford University for history, physical examination and baseline laboratory studies. On day 0 of the protocol, patients were infused with F.IX concentrate to bring factor levels up to ~100%, and, under ultrasound guidance, vector was injected percutaneously into 10–12 sites in the vastus lateralis of either or both anterior thighs. Injectate volume at each site was 250–500 μl, and sites were at least 2 cm apart. Local anaesthesia to the skin was provided by ethyl chloride or eutectic mixture of local anaesthetics (EMLA). To facilitate subsequent muscle biopsy, the skin overlying several injection sites was tattooed and the injection coordinates recorded by ultrasound. We observed patients in the hospital for 24 h after injection; routine isolation precautions were observed during this period to minimize any risk of horizontal transmission of vector. Patients are then discharged and seen daily in outpatient clinic for the next three days, then weekly at the home haemophilia centre for the next eight weeks, twice monthly up to five months, monthly for the remainder of the year, then annually in follow-up. Patients are instructed to infuse factor as usual for haemorrhagic episodes.

Laboratory studies. Laboratory studies drawn in follow-up included F.IX level, aPTT, Bethesda assay, anti-AAV neutralizing antibody titre, routine chemistries, muscle enzymes, CBC, urinalysis, HIV viral load for HIV positive patients, fragment 1.2, and collection of serum, semen, urine, saliva and stool for PCR detection of vector sequences. Patients underwent muscle biopsy of injected sites at 2, 6 and 12 months after injection; studies on skeletal muscle included routine haematoxylin and eosin staining, immunohistochemical staining for F.IX expression, PCR for vector sequences on extracted DNA and Southern blot with a vector probe if adequate amounts of muscle DNA were available. Whenever possible, an effort was made to draw blood samples before factor infusion if a haemorrhagic episode required treatment. All studies were performed in routine clinical laboratories (at CHOP and Stanford) using CLIA-approved procedures, except F.IX ELISA, immunostaining of muscle for F.IX expression, anti-AAV neutralizing antibody titre, PCR of body fluids for vector sequences and western blot to detect anti-F.IX antibodies. We carried out F.IX ELISA as described[15]. For immunohistochemical staining, frozen muscle tissue was cryosectioned and stained using a goat anti-human F.IX antibody (Affinity Biologicals; 1:800 dilution) as described[1], except that a biotinylated horse anti-goat IgG was used as a secondary antibody (1:200 dilution) for immunoperoxidase staining using a kit (Vector Laboratories). Sections were counter-stained with Myers haematoxylin stain.

Antibody assays. We determined AAV neutralizing antibody titres by incubating an AAV vector expressing *lacZ* with serial dilutions of patient serum, then used this cocktail to transduce HEK 293 cells. We lysed cells after 24 h and assayed enzymatic activity using the o-nitrophenyl β-D-galactopyranoside (ONPG) assay[16]. Samples were read at OD_{420} to measure β-galactosidase activity; sera were scored as positive for neutralizing AAV antibodies if the OD_{420} was ≤50% that observed when rAAV-lacZ was pre-incubated with negative control mouse sera. Positive samples were titred; AAV neutralizing antibody titres are presented as dilutions that inhibit infection of rAAV-lacZ by 50% based on the ONPG assay. We carried out western-blot analysis to detect anti-F.IX antibodies. Purified human F.IX was electrophoresed on a 7.5% polyacrylamide gel and transferred to a nitrocellulose membrane using an electroblot system (Biorad). The membrane was incubated with a 1:1,000 dilution of the patient's serum sample as primary antibody and 1:10,000 dilution of anti-human IgG peroxidase conjugate using a chemiluminescent substrate (Pierce) as a detecting antibody.

Viral shedding. We used a PCR assay to detect vector sequences in body fluids (serum, urine, saliva, semen and stool) and biopsied muscle. The 5′ primer (5′–AGTCATCGCTATTACCATGG–3′) was derived from the CMV enhancer and the 3′ primer (5′–GATTTCAAAGTGGTAAGTCC–3′) from

intron 1 of human *F9*. Amplified vector sequence yields a PCR fragment of 743 bp. For each sample, a control reaction containing the sample to be tested spiked with vector plasmid (50 copies/μg DNA) was also run to establish that the sample did not inhibit the PCR reaction. For semen, 3 μg of DNA was analysed (1 μg in each of 3 separate reactions); for saliva and biopsied muscle, 1 μg; and for urine, serum and stool, DNA was extracted from a 1–2 ml volume and analysed. The sensitivity of the assay is 50 copies of vector sequence in 1 μg DNA.

Factor IX levels. We determined F.IX levels using an automated analyser (MDA, Organon-Teknika, or MLA-800, Medical Laboratory Automation). Plasma test samples were mixed with F.IX-deficient substrate (George King, Inc.), and results compared with the degree of correction obtained when dilutions of verify reference plasma were added to the same F.IX-deficient substrate. The reference curve was linear down to a lower limit of 0.3%.

The F.IX measurements reported here deserve comment, as the changes are small. Most clinical laboratories do not report a numerical value for clotting factor levels of <1%, but in preparation for this trial, the coagulation laboratories at CHOP and Stanford University Medical Center prepared detailed standard curves for F.IX, which were linear down to levels of ~0.3%. Most authorities would agree that an experienced clinical coagulation laboratory can distinguish between levels <1% and >1%. The values of >1% in patient A were actually repeated and verified by a third clinical laboratory. Thus it appears that these numbers represent an increase from the patient's true baseline, which was also verified to be <1% by three clinical laboratories before the beginning of the trial.

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Efficient retrovirus-mediated transfer of the multidrug resistance 1 gene into autologous human long-term repopulating hematopoietic stem cells

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Pre-clinical studies indicate that efficient retrovirus-mediated gene transfer into hematopoietic stem cells and progenitor cells can be achieved by co-localizing retroviral particles and target cells on specific adhesion domains of fibronectin. In this pilot study, we used this technique to transfer the human multidrug resistance 1 gene into stem and progenitor cells of patients with germ cell tumors undergoing autologous transplantation. There was efficient gene transfer into stem and progenitor cells in the presence of recombinant fibronectin fragment CH-296. The infusion of these cells was associated with no harmful effects and led to prompt hematopoietic recovery. There was *in vivo* vector expression, but it may have been limited by the high rate of aberrant splicing of the multidrug resistance 1 gene in the vector. Gene marking has persisted more than a year at levels higher than previously reported in humans.

The successful transfer and expression of new genetic sequences in hematopoietic stem and progenitor cells may improve the management of both malignant and non-malignant human conditions[1–4]. Retrovirus-mediated gene transfer is widely used for this purpose because it has the potential for stable vector integration and expression[4]. Unfortunately, retrovirus-mediated gene transfer into adult human stem and progenitor cells has proven problematic, and clinical application of this therapy has been limited by low gene transfer into long-term repopulating cells[5–8] Several modifications have recently led to improved gene transfer into stem cells, including the use of newly discovered early acting cytokines, highly expressed alternative viral receptors, modified vectors, 'activated' bone marrow cells obtained after mobilization induced by growth factor, and co-localization of stem cells and vector particles by centrifugation or by transduction in the presence of autologous stroma or fibronectin[9–18].

We based our protocol on recent observations that fibronectin-mediated co-localization of target cells and retroviral particles increases gene transfer efficiency. Fibronectin contains specific adhesion domains for stem and progenitor cells and retroviral vectors and can increase the apparent titer of retroviral particles presented to target cells. CH-296, a chimeric molecule that contains these domains, has been associated with the highest effeciency of gene transduction[19]. Studies in nonhuman primates and a non-obese diabetic/severe combined immunodeficiency mouse model of human disease have demonstrated better gene transfer with CH-296 than with co-cultivation or vector supernatant infection alone[19,20].

For our trial, we enrolled patients undergoing autologous transplantation for germ cell tumors. These tumors have become a model for curable malignant disease, with more than 70% of patients with advanced disease cured by first line therapy. Patients with relapsed or refractory disease have a lower rate of being cured. The addition of high-dose carboplatin and etoposide plus rescue with autologous peripheral blood progenitor cells (PBPCs) successfully cures a substantial portion of relapsed patients. Moreover, the addition of three cycles of oral etoposide after transplantation further improves the outcome of patients with relapsed germ cell tumors[21]. However, myelosuppression has been a principal limitation, resulting in dose modification and treatment delay. The expression of the gene for human multidrug resistance 1 (MDR-1) in hematopoietic progenitor cells may render these cells resistant to oral etoposide[22,23], thus allowing for 'dose-intensive' therapy without delay, with the intention of further improving the survival rate of patients with germ cell tumors.

To begin to address this, we initiated a phase I study. To reduce

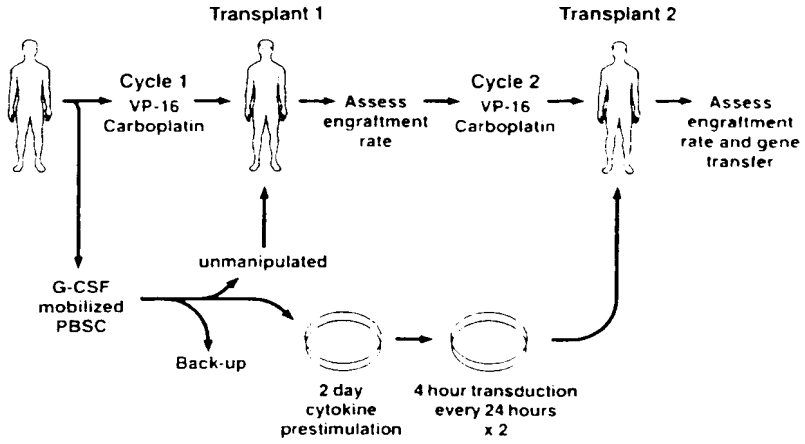

Fig. 1 Treatment protocol. Peripheral blood progenitor cells were mobilized using G-CSF. These cells were divided into three groups. The first was not manipulated and was infused after the first cycle of high-dose chemotherapy. The second was used for gene manipulation and was the sole source for hematopoietic reconstitution after the second cycle of high-dose chemotherapy. After CD34⁺ cell selection, cells were maintained in cytokine-containing media. Transduction was done on days 3 and 4 with clinical grade vector using CH-296. After etoposide (VP-16) transplantation, patients received three cycles of oral (not shown).

the risk that the MDR-1 might be inadvertently introduced into malignant cells[24], we first purified patients' peripheral blood stem and progenitor cells using the CD34 antigen, which is expressed on stem and progenitor cells but not on adult germ cell tumor cells[25]. Our study design allowed for the comparison of engraftment kinetics of unmanipulated and *ex vivo*-manipulated cells in each patient. In addition, the availability of a large database at our institution allowed comparison with a similar patient population that did not receive manipulated cells.

Here, autologous peripheral blood CD34⁺ cells transduced with MDR-1 in the presence of CH-296 engrafted in 12 patients undergoing autologous transplantation for germ cell tumor. There was not consistent MDR-1 expression, related in part to the presence of splicing signals in the MDR-1 vector, which may result in the generation of truncated, non-functional P glycoprotein[26,27]. However, the frequency of gene transfer, as measured by the presence of proviral-containing progenitors in patients' bone marrow and circulating white cells, was higher than in previously reported human trials of MDR-1. Moreover, vector-transduced hematopoietic cells derived from primitive progenitor cells persisted at high levels in most patients for at least 1 year. The transduction and transplantation protocol is a substantial advance in the use of retroviral vector to transduce human hematopoietic progenitor cells.

Patients and mobilization characteristics

We enrolled twelve patients eligible for tandem transplantation (median age, 29 years (range, 17–51); Fig. 1, treatment protocol). PBPCs were mobilized using 10 μg granulocyte colony-stimulating factor (G-CSF)/kg per day, and were divided into three groups. The first was unmanipulated and used for the initial transplant. The second was selected by CD34⁺, stimulated with cytokines, transduced with MDR-1 retroviral vector and then cryopreserved; this served as the only source of cells for the second transplant. The third, unmanipulated group was cryopreserved for use in case of graft failure. Mobilization was successful in 11 patients. For one patient, an insufficient number of cells was mobilized to meet the minimal requirements for this protocol, and the patient successfully completed tandem transplantation using unmanipulated PBPCs. One leukapheresis product from this patient was transduced; gene transfer data is included in this analysis.

CD34⁺ cell transduction

To facilitate transduction, CD34⁺ cells were maintained *ex vivo* in the presence of cytokines for 5 days. Cells were cultured with stem cell factor and interleukin 6 (SCF/IL-6) in the first ten patients, and with stem cell factor, megakaryocyte growth and differentiation factor and G-CSF (SCF/MGDF/G-CSF) in the final two patients. On days 3 and 4 of *ex vivo* stimulation, cells were cultured with MDR-1 vector on plates coated with CH-296 for 4 hours each day. After vector exposure, the cells were supplied with fresh media and cytokines and were incubated overnight on dishes coated with CH-296. Manipulated cells were cryopreserved on day 5. When CD34⁺ cells were cultured in the presence of SCF/IL-6 (10 patients requiring 17 separate transduction procedures), there was no consistent increase in cell number. In eleven procedures there was a loss in CD34⁺ cells of 4–62%, whereas there was a gain in CD34⁺ cells of 3–52% in six transductions. Changes in total cell numbers paralleled changes in CD34⁺ cell numbers using SCF/IL-6. In three separate transductions for the two patients in whom SCF/MGDF/G-CSF was used, there was an increase in total nucleated cells of 88% (range, 80–99%) and an increase in CD34⁺ cells of 39% (range, 30–42%).

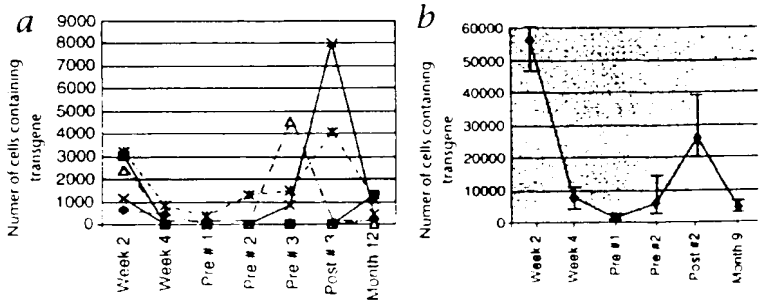

Fig 2 Transgene frequency in peripheral blood leukocytes as determined by quantitative real-time PCR. Values (as transgene-containing cells/10^6 cells) represent the median of three independent analyses. ***a***, Peripheral blood leukocytes from patients whose CD34⁺ cells were transduced in the presence of SCF/IL-6. ◆, patient 5; ■, patient 6; ▲, patient 8; ✕, patient 9, ★, patient 10. ***b***, Peripheral blood leukocytes from patient 12, whose CD34⁺ cells were transduced in the presence of SCF/MGDF/G-CSF. Pre #1, Pre #2 and Pre #3, samples obtained before first, second and third cycles of oral etoposide, respectively; Post #3, sample obtained 6–8 weeks after the last cycle of oral etoposide. Scales for vertical axes in *a* and *b* differ.

Table 1 Hematopoietic recovery

	Transplant 1 (Unmanipulated cells)	Transplant 2 (Transduced cells only)	Transplant 2 ('Historical' control)
CD34⁺ cell dose/kg	3.5×10^6 (1–9.7)	3.2×10^6 (2.3–5.7)	3.5×10^6 (1–14)
Days to ANC $> 0.5 \times 10^9$	10 (8–11)	9 (8–11)	11 (8–13)
Days to platelets $> 20 \times 10^9$	10 (10–14)	13 (10–35)	13 (8–36)

Both unmanipulated and transduced products were infused without toxicity. Data include the number of cells infused per kg body weight, and the number of days until the absolute neutrophil count (ANC) or platelet count reached more than 0.5×10^9 cells/l or 20×10^9 platelets/l, respectively (ranges, in parentheses). 'Historical' control, 25 consecutive patients with germ cell tumors treated in an identical way without receiving manipulated cells.

Assessment of early and late hematopoietic function

Eleven patients completed the tandem transplantation regimen; we used unmanipulated PBPCs during the first transplant, and MDR-1-transduced CD34⁺ cells for the second transplant. The median numbers of CD34⁺ cells/kg were 3.5×10^6 and 3.2×10^6 for the first and second transplants, respectively (Table 1). Both unmanipulated and transduced products were administered without infusion-related toxicity. Hematopoietic recovery rates after the first and second transplants were similar. The times to absolute neutrophil count above 0.5×10^9 cells/l were 10 days for the first transplant (unmanipulated cells) and 9 days for the second transplant (stimulated and vector-transduced cells). Platelet recovery was also similar for the first and second transplants. We compared the hematopoietic recoveries of these 11 patients with those of a control group of 25 consecutive patients with germ cell tumor treated in an identical way but not receiving manipulated cells. There were no differences in neutrophil or platelet recovery after CD34⁺ cell selection, cytokine stimulation, exposure to CH-296 and vector transduction (Table 1).

Complete blood counts of eight patients on samples obtained more than 1 year after infusion of cells transduced with MDR-1 showed all patients had normal hemoglobin levels, white blood cell counts and differentials (data not shown). Platelet counts were within normal range except for one patient, who had a slight reduction in platelets, to 103×10^9 platelets/l (normal, 150×10^9–450×10^9 platelets/l).

Gene transduction efficiency

We plated transduced cells in methylcellulose and counted progenitor colonies on day 14. We defined gene transfer efficiency as the percentage of progenitor colonies containing the MDR-1 vector, as assessed by PCR for individually isolated colonies. We screened a median of 52 colonies for each patient's transduced CD34⁺ cells (range, 27–87), which included a median of 30 erythroid burst-forming units (range, 6–53) and a median of 19 granulocyte–monocyte colony-forming units (CFU) (range, 8–36 colonies). The median gene transfer efficiency of all colonies immediately after transduction was 14% (range, 4–52%), and was 24% for erythroid burst-forming units (4–47%) and 13% for granulocyte–monocyte CFU (0–67%) (Table 2). To confirm the preclinical data demonstrating the drug selection potential of the A12M1 vector[28], we plated cells from patient 10 in the presence or absence of the anti-tumor agent pachtaxel at a concentration of 10 ng/ml. 'Control-transduced' CD34⁺ cells lacked the MDR-1 transgene, by PCR, and did not form colonies in the presence of paclitaxel. In contrast, 23% of transduced progenitors survived this exposure and all surviving colonies contained the transgene, by PCR analysis. Gene transduction efficiency (measured by PCR) in this patient was 52%, demonstrating that close to 50% of this patient's transduced colony-forming cells expressed functional MDR-1.

Engraftment of transduced cells

At 1 month after infusion, the median frequency of colonies containing the transgene in aspirated bone marrow was 12% (range, 0–78%). The percent of transduced progenitor cells in the bone marrow was maintained at 9% (range, 5–14%) and 8% (range, 3–10%) during the period of maintenance chemotherapy at 3 and 6 months after infusion, respectively. The percent of transgene-containing bone marrow progenitors was 0–15% in seven patients that could be evaluated at 12 months, with two patients demonstrating more than 10% colonies containing the transgene by PCR at that time (Table 3).

We used analyses of peripheral blood by nested PCR and quantitative real-time PCR to determine the presence of transgene in nucleated blood cells. Using nested PCR capable of detecting full-length and truncated MDR-1, we detected the presence of transgene in all 11 patients on day 14 after infusion of transduced cells. At 1 month after infusion, 8 of 11 patients had vector-containing cells in the peripheral blood. The results of nested PCR became negative in all patients in subsequent months; however, after the completion of maintenance chemotherapy, vector-containing cells reappeared in the circulation in four of nine patients. These four patients have maintained detectable levels (5–15%) of transgene-containing colonies in their bone marrow samples 1 year after infusion of cells.

To quantify the level of gene-transduced cells in the peripheral blood, we used real-time PCR with primers that detect the full-length MDR-1. We detected transgene-containing nucleated cells in seven patients up to 12 months after transplanta-

Table 2 Gene transduction efficiency

Patient	%(+) colonies	Total[a]	% BFU-E[b]	Total[b]	%CFU-GM[c]	Total[c]
1	15	27	33	6	12	17
2	34	47	33	33	36	14
3	31	36	28	25	50	8
4	4	50	7	29	0	18
5	6	83	7	51	13	32
6	8	87	12	53	3	36
7	12	52	13	30	9	22
8	5	60	4	28	0	21
9	9	50	12	26	7	29
10	52	58	47	43	67	15
11	31	52	29	41	31	13
12	27	60	37	30	17	30
Median	14	52	24	30	13	19

[a]Total number of colonies (granulocyte–monocyte CFU; granulocyte, erythroid, macrophage and megakaryocyte CFU, and erythroid burst-forming units) screened per patient. [b]Percent positive colonies and total erythroid burst-forming units analyzed. [c]Percent positive colonies and total granulocyte–monocyte CFU analyzed. BFU-E, erythroid burst-forming units; CFU-GM, granulocyte–monocyte CFU.

Table 3 Percentage of transgene-containing colonies in the graft and at 4 time points after transplantation

Subject	Graft	1 month	Pre cycle 2[a]	Post cycle 3[b]	1 year
1	15	ND	5	NA	NA
3	31	14	10	9	3
4	4	0	8	10	0
5	6	28	15	9	5
6	8	9	14	8	4
7	12	8	NA	NA	NA
8	5	14	7	4	15
9	9	8	NA	8	13
10	52	8	ND	3	7
11	31	78	NA	NA	NA
12	27	31	9	7	NA

[a]Timing for obtaining samples before cycle 2 of oral etoposide (VP16) (planned *in vivo* selection for transduced cells): 2 months after transplant of transduced cells in three patients, 3 months in three patients, and 5 months in 1 patients. [b]Timing for obtaining samples after cycle 3 of oral etoposide (VP16): 5 months in one patient, 4 months in two patients and 6 months in five patients. ND, not determined; NA, samples not available for analysis because of disease progression ($n = 5$) or patient refusal ($n = 3$) or because samples were not delivered ($n = 2$).

tion (Fig. 2). For the two patients (11 and 12) whose CD34$^+$ cells were pre-stimulated and transduced using SCF/MGDF/G-CSF, we detected 5.61% and 4.30% transgene-containing nucleated cells in the blood during the first month after transplantation. Maintenance chemotherapy for these two patients was delayed because of intervening surgical procedures to remove residual tumor masses. Patient 12 received only two cycles, because of disease progression. In this patient, transgene-containing cells decreased from 5.61% to 0.13% before *in vivo* exposure to etoposide. After the first and second cycles of oral etoposide, circulating transgene-containing cells increased to 0.58% and 2.60%, respectively, indicating an increase of 450% with each cycle. Patient 11 was not compliant with oral etoposide and received only part of the first cycle. The circulating transgene-containing cells continued to decrease with time in the absence of exposure to etoposide, from 4.3% immediately after transplantation to 0.1% 8 months later (data not shown). Patients 8, 9 and 10 had low levels of transduced cells (range, 0.1–0.3%) in the peripheral blood shortly after transplantation (Fig. 2*a*). After receiving oral etoposide, they showed an augmentation in the number of circulating transduced cells (an increase of 300–900% in transgene-containing cells). These data indicate *in vivo* selection of transduced cells.

The MDR-1 vector used here contained both truncated and full-length transgenes because of splice sites in the MDR-1 cDNA. To determine transgene integrity, we analyzed transduced cells and samples obtained after transplant by PCR using two primers sets that allow for the detection of either full-length or truncated MDR-1 integrated DNA. We detected both truncated and full-length versions in the transduced products (Fig. 3). Unexpectedly, PCR analysis of peripheral blood samples on day 14 showed only the truncated transgene in 6 out of 11 patients. The ratio of full-length transgene to truncated transgene isoforms during the first month after transplantation in four separate analyses from patient 12 was 1:2–1:10.

To determine whether a functional P-glycoprotein was present at later times, we cultured bone marrow cells with or without paclitaxel. Cryopreserved samples from five patients at 1 year and a sample from one patient at 9 months (time of disease progression) were available for analysis. We detected no colonies in three patients and five control samples grown in the presence of paclitaxel. In the other three patients (patients 8, 10 and 12), 4, 2 and 6 colonies per 2×10^5 bone marrow cells respectively, grew in the presence of paclitaxel, whereas we counted 32, 8 and 40 colonies per 2×10^5 bone marrow cells respectively, in the absence of paclitaxel. All colonies grown in the presence of paclitaxel contained vector DNA, as shown by PCR. These data indicate the presence of a functioning transgene in these patients 1 year after transplantation.

Clinical outcome

The median follow-up at this analysis was 18 months from the infusion of transduced cells. Three of the eleven patients have died from progressive germ cell tumors. One patient relapsed and obtained a remission using further chemotherapy. Eight patients are alive with normal marrow cellularity and function when assessed at 1 year after transplantation. Of the 33 planned cycles of maintenance etoposid, 28 were given. Only one was withheld because of prolonged cytopenia. Four were withheld because of disease progression. In addition, one patient required early termination of cycle 3 because of neutropenic fever.

We assessed treatment-related toxicity by frequent clinical and laboratory evaluations. So far, there have been no unanticipated adverse clinical consequences. We detected neither viral envelope nor replication-competent retroviruses in transduced CD34$^+$ cells before infusion or in any samples obtained after transplantation. We have used both enzyme-linked immunosorbent assay (ELISA) and western blot analysis to assess the development of antibodies against CH-296. We obtained samples eight times during the first year after infusion of transduced cells. We also obtained samples before transplantation for baseline analysis. We re-assayed, using western blot analysis, five samples that exceeded the established ELISA titer limit. Of these, one sample from patient 3, obtained 1 year after transplantation, produced weakly positive results. The other eight samples from this patient produced negative results by ELISA and western blot analysis. No sample before transplantation was available from this patient, who has no clinical symptoms and is in good health 28 months after infusion of transduced cells.

Discussion

Transplantation of cytokine-stimulated CD34$^+$ cells transduced with AM12 MDR-1 retroviral vector in the presence of recombinant fibronectin CH-296 was associated with prompt hematopoietic recovery and no adverse events. With more

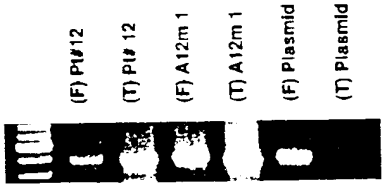

Fig. 3 Transgene integrity. Samples from transduced CD34$^+$ cells from patient 12 (Pt#12), producer cell line (A12m 1) and plasmid containing MDR 1 cDNA (Plasmid) were analyzed by PCR for the presence of truncated (T) and full-length (F) MDR-1. As expected, the plasmid contains only full-length MDR-1, whereas A12m 1 and the patient's sample contain both full-length and truncated transgene.

than 1 year of follow-up, we have demonstrated the continued contribution of vector-derived cells to hematopoiesis. The level of long-term engraftment of retrovirally transduced peripheral blood progenitor cells was the highest reported so far.

Here, the exposure of CD34$^+$ cells to the A12M1 vector with CH296 yielded efficient gene transfer. The A12M1 vector has been used before with less-impressive gene transfer efficiencies. In another study[5], five patients were transplanted with a mixture of untransduced and CD34$^+$-enriched stem cells transduced on plates coated with whole fibronectin (not CH-296). The latest time at which vector-transduced cells were detected by PCR was 10 weeks after transplantation[5]. There was only a transient and low rate of engraftment of MDR-1-transduced CD34$^+$ cells in 5 of 20 patients using either suspension or stroma methodology[6]. Stroma was also used to aid gene transfer in a study of six patient; half of the enriched CD34$^+$ cells were transduced with an MDR-1 vector and the other half with a marking vector (NeoR) (ref. 29). There was low-level gene marking of granulocytes (0.01–1%, by semi-quantitative PCR), with one of these six patients showing NeoR marking and two patients showing MDR-1-marked-granulocytes close to 6 months after transplantation[29]. In a second study, four patients received CD34$^+$ cells transduced with the MDR-1 vector along with unmanipulated cells[30]. One patient had detectable MDR-1 transgene in granulocytes (by semi-quantitative PCR) at almost 4 months; no long-term data was provided[30]. The ultimate goal of this gene therapy approach is decreased cytopenia after chemotherapy administration. Although the patients in our study seem to have tolerated after transplant chemotherapy better than patients in other studies have, the possibility of patient selection bias cannot be excluded and therefore the contribution of transduced cells to the clinical outcomes is unclear[21].

Improved transduction efficiency in the presence of matrix proteins is well-described, and the advantages of CH-296-assisted gene transfer include ease of use and safety[20,31–33]. This approach does not pose the technical problems associated with autologous or allogeneic stroma[34]. Also, recent data indicate the preservation of long-term repopulating cells in *ex vivo* cultures that contain matrix protein such as fibronectin[35]. Transplantation models in nonhuman primates have noted improved transduction of long-lived hematopoietic progenitor cells with results similar to our findings in humans. In one study, the frequency *in vivo* of gene marked cells derived from a long-lived CD34$^+$ progenitor cell transduced in the presence of CH-296 was almost one log higher than that of those transduced by co-culture[20]. In a preliminary report[36], two patients with severe combined immunodeficiency were treated on a gene therapy trial using CH-296 and a retroviral vector expressing the common gamma chain of interleukin 2 receptor. There was substantial correction of the disease phenotype in lymphoid elements of both patients[36]. Our data, in addition to many other studies (including those in nonhuman primates), indicate CH-296 may be involved in the increased gene transfer reported here.

The optimum cytokine combination for increasing gene transfer into human stem and progenitor cells remains to be defined. SCF/MGDF/G-CSF has been shown to yield a reproducibly higher level of gene transduction of progenitor cells compared with that of CSF/IL-6 (ref. 32). Accordingly, we amended our protocol, and the CD34$^+$ cells from the last two patients were transduced using SCF/MGDF/G-CSF. These two patients had the highest gene marking of bone marrow colonies one month after transplant and the highest levels of transduced cells in the peripheral blood, and these cells persisted throughout the observation period (9 months). Unfortunately, the two patients relapsed approximately 7 months after transplantation, preventing longer follow-up. Alternate cytokine combinations may provide further improvements. The addition of FLT-3 ligand to IL-3/IL-6/SCF improves gene transfer in nonhuman primates[37]. There was similar long-term marking of peripheral blood cells in a canine study using G-CSF/SCF/ FLT-3 ligand and CH-296 (ref. 38).

Our data demonstrate that exposure of CD34$^+$ cells to CH-296 did not adversely affect engraftment kinetics. Hematopoietic function has remained normal in the eight surviving patients with almost 2 years of follow-up. This is an encouraging finding, given a recent report on the development of myeloproliferative syndrome in mice transplanted with MDR-1-transduced marrow cells[39]. At present, we do not know whether expression of MDR-1 or other factors led to the hematologic disorder[39]. However, our study differs in several ways from that mouse study[39]. A mutated version of the MDR-1 cDNA that contains a substitution of valine with glycine at position 185 was used in the mouse study, and the transduced cells were expanded *in vitro* for a much longer time (up to 12 days)[39]. Multiple integrations may have occurred as a result of this transduction procedure, increasing the risk of insertional mutagenesis[31]. In contrast to that study, abnormal hematopoiesis was not reported in four human studies using MDR-1 or in many other mouse transplant experiments using the A12M1 vector[5,6,29,30,40]. As prior human gene therapy studies of MDR-1 attained only lower levels of transduction and our study showed that most transduced cells contained a truncated MDR-1 gene, the propensity of MDR-1 vectors to elicit human myeloproliferative disorders remains to be determined.

In conclusion, we have demonstrated safe and efficient retrovirus-mediated gene transfer into cytokine-stimulated, CD34$^+$ hematopoietic cells transduced using CH-296. Our findings are relevant to most gene therapy approaches targeting hematopoietic cells and indicate therapeutic levels of gene-transduced cells can be attained for a variety of genetic and malignant diseases.

Methods

Patient selection. Patients with documented germ cell tumors who had refractory or relapsed disease enrolled on this study. Eligibility criteria included an Eastern Cooperative Oncology Group performance status of 2 or less, normal cardiac function, normal liver function tests, creatinine clearance of more than 60 ml/min, diffusion capacity of more than 50%, and absence of active infections. All patients met the pre-transplant assessment criteria according to our institutional protocol and signed an informed consent form conforming to our institutional review board guidelines.

Mobilization and Isolation of CD34 cells. PBPCs were mobilized using 10 μg G-CSF/kg per day (Amgen, Thousand Oaks, California) subcutaneously for 4 d. Leukapheresis was initiated using the mononuclear cell collection procedure of the COBE Spectra Cell Separator (COBE Laboratories, Lakewood, Colorado). Leukapheresis goals were a minimum of 2×10^7 CD34$^+$ cells/kg for gene transfer and 1×10^8 mononuclear cells/kg for the first transplantation and for 'back-up' to be used in case of graft failure of the transduced cells. A maximum of 5 d of leukapheresis were allowed. The Isolex® 300i system (Nexell Therapeutics, Irvine, California) was used to process PBPCs and select CD34$^+$ cells according to the manufacturer's recommendations.

Retroviral vector and transduction protocol. Genetix Pharmaceuticals (Cambridge, Massachusetts) provided the AM12M1 vector. This vector con-

tains the MDR-1 cDNA within the Harvey murine sarcoma virus long terminal repeats and was packaged by the AM12 amphotrophic packaging cell line. The protocol has received approval from the National Institutes of Health Recombinant DNA Advisory Committee and the Food and Drug Administration, and the vector used in this trial was produced at the National Gene Vector Laboratory (Indianapolis, Indiana). Cells were cultured in Iscove's Modified Dulbecco's Media (IMEM, BioWhittaker, Walkersville Maryland) with 10% fetal calf serum (Hyclone, Logan Utah) containing SCF/IL-6 (R&D Systems, Minneapolis, Minnesota) or G-CSF/MGDF/SCF (at final concentration of 100 ng/ml each) (Amgen, Thousand Oaks, California). The final cell concentration was 5×10^5–1×10^6 cells/ml. After 48 h of prestimulation, 4×10^7 CD34$^+$ cells were plated on non-tissue culture dishes treated with the fibronectin fragment CH-296 (Takara Shuzo, Otsu , Japan). Cells were exposed to vector for 4 h, then collected and resuspended in fresh media with cytokines; the next day, the transduction was repeated and the cells were cultured for an additional 8–16 h before being cryopreserved. A portion of the transduced cells were removed before the cryopreservation to document vector integration, expression of MDR-1 and the absence of replication-competent retrovirus.

Transplantation regimen. Patients received etoposide (750 mg/m^2 per day, intravenously over 2 h) and carboplatin (700 mg/m^2 per day, intravenously over 30 min) on days 6, 5 and 4 before the infusion of stem cells (Fig.1). Patients also received prophylactic quinolone antibiotics and fluconazole. Frozen cell products were brought to the patient's room, thawed, and infused without delay. All patients received 5 µg G-CSF/kg per day until absolute neutrophil counts were 2×10^9 cells/l for 2 consecutive days. After hematologic recovery from cycle 2 (3×10^9 or more white blood cells/l; absolute neutrophil counts, 1.5×10^9 or more cells/l; and 75×10^9 or more platelets/l), Etoposide was administered orally at a dose of 50 mg twice a day for 21 consecutive days every 4 weeks for three cycles. If absolute neutrophil counts decreased to less than 0.5×10^9 cells/l, then etoposide was stopped and the doses of the following cycles were reduced by 25%. Subsequent cycles were started when absolute neutrophil counts were more than 1.5×10^9 cells/l and platelet counts were more than 75×10^9 platelets/l.

Hematopoietic progenitor assays and drug resistance. PBSCs or bone marrow cells (5×10^2 for CD34-selected and 5×10^4 for marrow cells) were 'seeded' in plastic 35-mm tissue cultures dishes containing 1.1% methylcellulose (Stem Cell Technologies, Vancouver, Canada). Media contained 1 ml 30% FBS, 50 µM β-mercaptoethanol, cytokines (50 ng/ml SCF; 10 ng/ml GM-SCF, 10 ng/ml IL-3 and 3 U/ml erythropoietin). Cultures were incubated at 37 °C in an atmosphere of 100% humidity and 5% CO_2. Erythroid burst-forming units, granulocyte–monocyte CFU, and granulocyte, erythroid, macrophage and megakaryocyte CFU were counted *in situ* after 14 d. Paclitaxel was used at a dose of 10 ng/ml for the drug-resistance assay (Bristol-Myers Squibb, Princeton, New Jersey). Total cellular DNA was isolated from individual colonies on day 14 for PCR analysis. Colonies were assigned scores, and then DNA was isolated for PCR. Each colony was placed into 150 µl PBS (Sigma), 150 µl phenol–chloroform–isoamyl alcohol (Roche Diagnostics, Indianapolis, Indiana) was added, and each tube was vortexed well. Samples were incubated on ice for 15 min, and then centrifuged at 12,000*g* for 10 min at 4 °C. The upper aqueous phase was transferred to a clean tube containing 500 µl isopropanol (Sigma). DNA was precipitated overnight at –20°C, and then samples were centrifuged at 12,000*g* at 4 °C for 20 min to pellet DNA; the pellets were washed with 70% ethanol. Pellets were air-dried and then resuspended in 30 µl of water. Care was taken to exclude adjacent colonies to prevent cross-contamination and overestimation of gene transfer efficiency by nested PCR. To confirm the validity of this method, we mixed MDR-1-transduced CD34$^+$ cells and cytokine-stimulated control CD34$^+$ cells at various ratios. These cells were then plated in methylcellulose, and the gene transfer efficiency was calculated for each ratio. As the percent of MDR-1-transduced CD34$^+$ cells decreased, there was a corresponding decrease in the number of transgene-containing colonies. Many colonies were screened per patient, and only those with a sufficient amount of recovered DNA, as judged by the presence of β-globin DNA, were included in the calculation of gene transfer efficiency.

Nested and quantitative PCR. PCR reactions used 10 µl DNA in a total volume of 50 µl per reaction containing 2 U Amplitaq DNA polymerase (Perkin Elmer, Foster City, California). Both rounds of MDR-1 PCR consisted of 30 cycles of 94 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min. Primers for the first round were 5′–GCCCACATCATCATGATC–3′ and 5′–GTCTCCTACTTTAGTGCT–3′ (494-base-pair product)[45]. The second round used primers 5′–ACGGAAGGCCTAATGCCG–3′ and 5′–TGACTCGATGAAGGCATG–3′ (414-base-pair product). As a control for the presence of colony DNA, PCR was used to assess the presence of the β-globin gene with the primers 5′–GAATCCAGATGCTCAAGGCC–3′ and 5′–CAATCCAGCTACCATTCTGC–3′ (344-base-pair product). For nested PCR, 1×10^4–5×10^4 bone marrow or peripheral blood cells were lysed using 0.6 ml Cell Lysis Solution (Puregen, Gentra Systems, Minneapolis, Minnesota). For quantitative PCR, TaqMan Multiplex PCR (PE Applied Biosystems, Foster City, California) for both MDR1-cDNA and ApoB gene was used. DNA (0.5 µg) was plated in a 96-well optical tray with optical caps (MicroAmp; Perkin Elmer, Norwalk, Connecticut). The final reaction mixture of 50 µl consisted of 1x TaqMan buffer A (50 mM KCl, 10 mM Tris HCl, pH 8.3, 0.01 mM EDTA and 60 nM Passive Reference rhodamine carboxyl X), 5.5 mM $MgCl_2$; 300 µM dATP, dCTP and dGTP, 600µM dUTP; 300 nM 5′ primer MDR1-F (5′–AGGAAGCCAATGCCTATGACTTTA–3′); 300 nM 3′ primer MDR1-R (5′–AATGGCGATCCTCTGCTTCTG–3′); 200 nM fluorescent probe TP-MDR1 (5′–carboxyfluorescein–CATGAAACTGCCTCATAAATTTGACACCCTG–N,N,N′,N′-tetramethyl-6-carboxyrhodamine–3′); 80 nM 5′ primer ApoB-F (5′–TGAAGGTGGAGGACATTCCTCTA–3′); 80 nM 3′ primer ApoB-R (5′–CTGGAATTGCGATTTCTGGTAA–3′); 200 nM fluorescent probe TP-ApoB (5′(VIC, –CGAGAATCACCCTGCCAGACTTCCGT–N,N,N′,N′-tetramethyl-6-carboxyrhodamine–3′;; 0.5 U AmpErase UNG (uracil-N-glycosylase), and 1.25 U AmpliTaq Gold DNA Polymerase (Perkin Elmer, Norwalk, Connecticut). The set of primers and probes that detect both full-length and truncated transgene were MDR-F3 (5′–GAAGAAGGGCCAGACCGCTG–3′), MDR-R3 (5′–ACAGGATGGGCTCCTGGG–3′) and TP-MDR3 (5′–carboxyfluorescein–CAGTGGCTCCGAGCACACCTGG–N,N,N′,N′-tetramethyl-6-carboxyrhodamine–3′). All samples were assayed in triplicate. The standard curves were of hematopoietic cell lines (KG1 and U937) transduced with A12M1 and MDR-1. PCR amplification used an ABI PRISM™ Sequence Detection System (PE Applied Biosystems, Foster City, California) and the following parameters: 50 °C for 2 min (UNG incubation), 95 °C for 10 min to activate the *Taq* DNA polymerase, then 50 cycles of 95 °C for 15 s (denaturation) followed by 60 °C for 1 min (annealing and extension).

Safety assessment. The safety of the retrovirus-mediated gene transfer was assessed by analysis for the presence of both replication-competent retrovirus and viral envelope. Mus dunni cells (1×10^8) were co-cultured for 3 weeks, then tested by the sarcoma-positive/leukemia-negative (S$^+$/L$^-$) assay[47]. Results were available before the infusion of transduced cells. PCR for viral envelope was done on all transduced products and after transplantation samples as described[44].

Antibodies against CH-296 were tested by analysis of patient sera samples by immunoassay at an independent commercial facility (Tanox, Houston Texas). Takara Shuzo (Otsu, Japan) developed both ELISA and western blot analyses for detecting antibodies against CH-296, and transferred the standard operating procedure to Tanox (Houston, Texas). Samples were considered positive if they exceeded ELISA limit and were confirmed by western blot analysis. Each patient serum sample was diluted 1:11. A mouse monoclonal antibody against CH-296 was used as the positive control. Samples from normal human serum (WAKO) were used as the negative control. Samples with ratio exceeding the mean + 3 s.d. of the negative control on two separate analyses were assigned scores as potential positives and were subjected to confirmatory western blot analysis. For western blot analyses, CH-296 was reduced and denatured, then separated by standard SDS–PAGE and blotted into a PVDE membrane. This membrane was blocked with horse serum and divided into three pieces. One was incubated with the mouse antibody against CH-296; a second, with WAKO at 1:11 dilution; and a third, with the sample at a dilution of 1:11. The expected band size for CH-296 was 63 kDa.

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organic carbon would thus be degraded at a rate of (0.88 to 1.50) $\times 10^{-4}$ gC cm^{-3} $year^{-1}$. As a consequence, even sapropels consisting entirely of organic carbon (~0.9 gC cm^{-3}) would be completely degraded within 10,000 years. Organic carbon compounds in sapropels have been preserved over much longer time intervals, although a high fraction of microbial cells in the sapropels are physiologically active and continue to use organic carbon originating from the sapropels. The above comparison thus indicates that prokaryotes in sapropels have significantly lower maintenance energy requirements than any of the pure cultures investigated to date.

Mediterranean sapropels harbor large populations of previously unknown members of the green nonsulfur bacteria and crenarchaeota. Our cumulative evidence suggests that these prokaryotes are physiologically active, are specifically adapted to the specific conditions as they prevail in sediments with large amounts of subfossil kerogen, and are capable of altering the organic matter in situ even 217,000 years after its deposition.

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Supporting Online Material

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Materials and Methods
Figs. S1 to S3

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Correction of ADA-SCID by Stem Cell Gene Therapy Combined with Nonmyeloablative Conditioning

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Hematopoietic stem cell (HSC) gene therapy for adenosine deaminase (ADA)–deficient severe combined immunodeficiency (SCID) has shown limited clinical efficacy because of the small proportion of engrafted genetically corrected HSCs. We describe an improved protocol for gene transfer into HSCs associated with nonmyeloablative conditioning. This protocol was used in two patients for whom enzyme replacement therapy was not available, which allowed the effect of gene therapy alone to be evaluated. Sustained engraftment of engineered HSCs with differentiation into multiple lineages resulted in increased lymphocyte counts, improved immune functions (including antigen-specific responses), and lower toxic metabolites. Both patients are currently at home and clinically well, with normal growth and development. These results indicate the safety and efficacy of HSC gene therapy combined with nonmyeloablative conditioning for the treatment of SCID.

Gene therapy trials have demonstrated the safety and feasibility of engineering hematopoietic stem cells (HSCs) for treating inherited hematopoietic diseases (*1*–*6*). In these studies, however, the frequency of multipotent genetically modified HSCs and the levels of long-term transgene expression were variable, with limited clinical effect. This variability could be influenced by vector design, gene transfer protocols, or inadequate engraftment and expansion of genetically corrected HSCs. Recent improvements in HSC gene transfer, combined with a strong selective advantage for growth and differentiation of lymphoid cells, allowed investigators to correct the immune defect in the SCID variant due to γ-chain deficiency (SCID-X1) (*7*).

In ADA-SCID the purine metabolic defect (*8*) leads primarily to impaired lymphocyte development and function but also to nonimmunological abnormalities, which indicates that this disease is more complex than other SCIDs (*8*–*10*). The accumulation of toxic metabolites may offer a selective advantage to cells that produce sufficient vector-derived ADA. In previous gene therapy trials, this advantage might have been lost because of simultaneous treatment with bovine enzyme [polyethylene glycol–conjugated ADA (PEG-ADA)] replacement therapy. Recent experience with an ADA-SCID patient treated with transduced peripheral blood lymphocytes (PBL) (*11*, *12*) shows that PEG-ADA discontinuation results in preferential expansion of T cells containing the ADA gene capable of sustaining immune functions, but it did not completely correct the metabolic defect (*12*). These data suggest that, for long-term full

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clinical benefit, correcting the metabolic defect could be as important as correcting the immune defect.

We recently developed an improved gene transfer protocol into $CD34^+$ HSCs (*13–15*), which allows efficient transduction while preserving differentiation capacity into multiple lineages, including myeloid cells, B cells, natural killer (NK) cells, and T lymphocytes, as shown by in vitro and in vivo assays (*15*). We applied this protocol to two ADA-SCID patients (Pt1 and Pt2), who lacked an HLA-identical sibling donor and for whom PEG-ADA was not available (*13*). To provide an initial developmental advantage to transduced HSCs and create space in the bone marrow (BM), we treated the patients with a low-intensity, nonmyeloablative conditioning regimen (*13*). This allowed us to fully exploit the selective advantage of genetically corrected cells and to evaluate the clinical efficacy of gene therapy.

Pt1 and Pt2 were enrolled in the gene therapy trial at 7 months and at 2 years and 6 months of age, respectively (*13*). Autologous $CD34^+$ cells were collected from BM (Pt1, 4.15×10^6 cells per kg of body weight; Pt2, 1.08×10^6 cells per kg of body weight), transduced with GIADAI retroviral vector, and infused 4 days later (*13*). Pt1 received 8.6×10^6 $CD34^+$ cells per kg, containing 25% transduced colony-forming units in culture (CFU-C), and Pt2 received 0.9×10^6 $CD34^+$ cells per kg, with 21% transduced CFU-C. At days −3 and −2, both patients received nonmyeloablative conditioning with busulfan (2 mg per kg per day). Neither patient experienced toxicity nor required blood component transfusion. After a transient myelosuppression (neutrophil nadir: Pt1, day +17, 0.15×10^3 cells per μl, Pt2, day +19, 0.4×10^3 cells per μl; platelet nadir: Pt1, day +31, 154×10^3 cells per μl; Pt2, day +30, 23×10^3 cells per μl), hematopoiesis recovered as expected [days to absolute neutrophil count (ANC) ≥ 500 cells per μl: Pt1, 22 days; Pt2, 21 days] (Fig. 1, A and B). Pt1, whose pretreatment ANC was already low, experienced 12 days of ANC < 500 and then recovered to normal levels (Fig. 1A), whereas Pt2 experienced a single day of ANC < 500 (Fig. 1B). In Pt1, who has a follow-up of 14 months, the number of PBL increased progressively from <100 per μl to 2000 per μl at day +150, a level that was maintained throughout the remaining follow-up (Fig. 1C). Within the lymphocyte subsets, the first increase occurred in B cells and NK cells, followed by T cells (day +90) (Fig. 1E) (*13*). T cells developed normally into both $CD3^+$ $CD4^+$ cells and $CD3^+/CD8^+$ subsets (Fig. 1G) and expressed a normal pattern of activation markers. Restoration of thymic activity was demonstrated by the dramatic increase in $CD4^+/CD45RA^+$ naive T cells and T cell receptor excision circles (TREC) (*16*) in $CD3^+$ cells to the levels observed in age-matched controls (Fig. 1G) (*13*). Gene therapy led to normalization of proliferative responses to polyclonal stimuli [CD3 monoclonal antibody (anti-CD3 mAb), with or without anti-CD28 mAb, phytohemagglutinin (PHA), pokeweed mitogen, and concanavalin A] and, more importantly, to nominal antigens (candida, tetanus toxoid) (Fig. 2A) (*13, 17*). Proliferative responses and cytotoxic activity to alloantigens were normal. The T cell receptor variable region β chain repertoire evaluated by polymerase chain reaction (PCR) heteroduplex analysis (*18*) showed a normal heterogeneous pattern. Serum immunoglobulin M (IgM), immunoglobulin A (IgA), and immunoglobulin G (IgG) increased to normal levels, which allowed us to discontinue intravenous immunoglobulin (IVIG) 6 months after gene therapy (Fig. 2A), and we detected isohemagglutinins for the first time (1:8 to 1:16). After vaccination with tetanus toxoid, both T cell proliferative responses and specific antibody levels in the serum were comparable to those of age-matched controls (Fig. 2A). A follow-up of 12 months is available for Pt2. In this patient, lymphocytes increased to 400 cells per μl, with slower kinetics than Pt1 (Fig. 1D). The increase occurred mostly in the T cell subset (Fig. 1F), as indicated by a significant increase in TREC (Fig. 1H). Gene therapy led to a substantial increase in proliferative responses to polyclonal stimuli (Fig. 2B). T cell lines generated ex vivo 6 months after gene therapy proliferated normally in response to interleukin 2 and anti-CD3 mAb, with or without anti-CD28 mAb.

Several findings support the hypothesis that Pt2 is also reconstituting B cell functions: (i) normalization of serum IgM and IgA was achieved by the fourth month after gene therapy (Fig. 2B); (ii) the patient maintained IgG levels above 800 mg dl in response to a delayed schedule of IVIG, which suggests endogenous IgG production; (iii) before gene therapy and despite IVIG, the patient was affected by recurrent respiratory infections, chronic diarrhea, and scabies. Twelve months after gene therapy, the patient showed no sign of respiratory infections and scabies and recovered normally from two transient episodes of diarrhea.

We next analyzed by quantitative real-time PCR (Q-PCR) (*13*) the proportion of vector-containing cells in purified subpopulations of PB and BM from both patients at

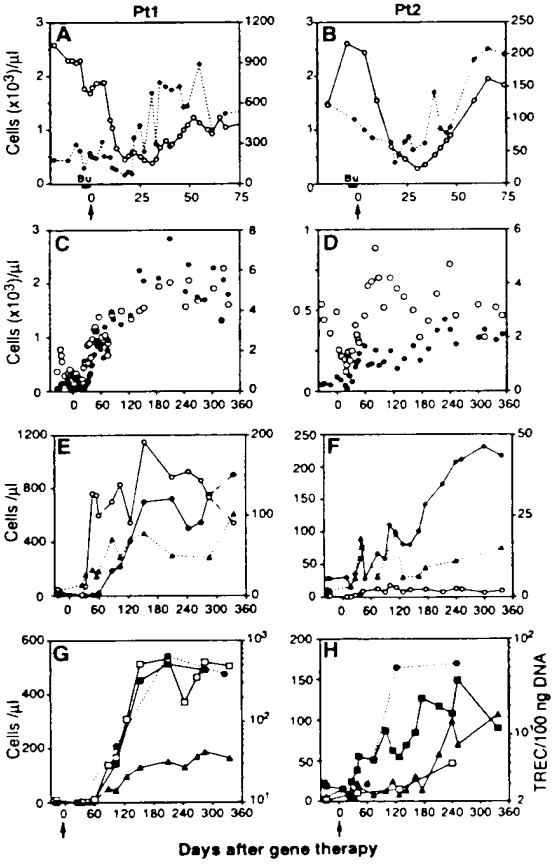

Fig. 1. Hematopoietic and lymphoid reconstitution after gene therapy. (**A** and **B**) Absolute neutrophil counts (solid diamonds) (left ordinate) and platelet counts (open circles) (right ordinate) of Pt1 (left column) and Pt2 (right column) before and after gene therapy. Bu indicates the 2 days of busulfan administration; arrows show the date of infusion of transduced $CD34^+$ cells (day 0). (**C** and **D**) Total lymphocyte counts (solid circles) (left ordinate) and white blood cell counts (open circles) (right ordinate) in the PB. (**E** and **F**) Absolute counts of PB $CD19^+$ B cells (open circles), $CD3^+$ T cells (solid diamonds) (left ordinate), and $CD56^+/CD16^+$ NK cells (solid triangles) (right ordinate). (**G** and **H**) Absolute counts of PB $CD3^+/CD4^+$ T cells (solid squares), $CD3^+/CD8^+$ T cells (solid triangles), $CD4^+/CD45RA^+$ naive T cells (open squares), and numbers of TREC (solid circles) in $CD3^+$ cells. The scale for TREC numbers is on the right. TREC levels in age-matched controls are 270 ± 130 copies per 100 ng of DNA.

different time points after gene therapy (Fig. 3). Vector-containing cells were detected first in granulocytes as early as 3 weeks in Pt1 and at 2 weeks in Pt2. Both patients showed genetically corrected cells in multiple lineages, including granulocytic, erythroid, megakaryocytic, and lymphoid cells, which were detected at higher levels in Pt1. The frequency of vector-containing cells was higher in lymphoid subsets (Fig. 3, C, D, G, and H) than in the other lineages (Fig. 3, A, B, E, and F), which indicates a stronger selective advantage for differentiation of genetically corrected B, NK, and T cells. We also observed an initial increase in untransduced cells (in Pt1 from 10 $CD3^+$ cells per μl before gene therapy to 220 cells per μl at day 100, with 70% untransduced cells), which could be related to a beneficial effect of systemic detoxification mediated by ADA-producing transduced cells.

In Pt1, the frequency of vector-containing T cells increased progressively and reached 70% at 11 months of follow-up (Fig. 3C). At the same time, virtually all NK cells present in PB and in BM were transduced (Fig. 3C). Transduced B cells were first detected in the BM at day +30 and 1 month later in PB (Fig. 3D). Strikingly, the frequency of transduced B cells was higher in the PB than in the BM, which suggests a preferential differentiation of genetically corrected cells and/or a growth advantage for peripheral B cells. The observation that BM immature B cells (surface IgM^+) contained a higher frequency of transduced cells (17%) than pre-B cells (8.5%) confirmed this hypothesis. In Pt2, genetically engineered $CD3^+$ T cells appeared later than in Pt1, but the frequency of these cells progressively increased up to 100% at day +240 (Fig. 3G). Persistent production of genetically corrected granulocytes, monocytes, megakaryocytes, and erythroid cells was observed, with levels ranging from 5 to 20% (Fig. 3, A and B) in Pt1, demonstrating the engraftment of multipotent HSCs. This conclusion was further supported by the consistent finding of neomycin (Neo)-resistant CFU-C (6.5% at day +330), which was confirmed by PCR analysis of individual CFU-C and by Q-PCR of purified BM $CD34^+$ cells (11% at day +330) (Fig. 3A).

To prove that genetically corrected HSCs retained their repopulation and differentiation properties, we isolated $CD34^+$ cells from the BM of Pt1 at day +330 after gene therapy and tested them for their lymphoid differentiation capacity. $CD34^+$ cells plated in vitro into a B/NK differentiation assay were able to generate B and NK cells that contained 4 and 9% of transduced cells, respectively (*15*). BM $CD34^+$ cells were also transplanted into the BM/thymus of SCID-hu mice (*19*) and analyzed after 8 weeks. Donor cells (identified by HLA-specific mAb) engrafted in the BM/thymus and differentiated into transduced mature B and T cells (frequency of transduced $CD19^+$ cells by Q-PCR, range 0.3 to 15.2%; $CD3^+$ cells, range 0.14 to 31.2%). These data formally demonstrate that engineered HSCs retained their ability to reconstitute human hematolymphopoiesis in vitro and in vivo in a secondary transplant 11 months after infusion.

Biochemical studies (*13*, *20*) demonstrated that gene therapy completely restored intracellular ADA enzymatic activity in PBL (Fig. 4A) and in BM $CD19^+$ B cells (590 units versus 300 ± 100 units in normal controls). Indeed, vector ADA was expressed at the mRNA level in differentiated cells of Pt1, as assessed by reverse transcriptase (RT)-PCR analysis in T cells, B cells, granulocytes, and monocytes (*17*). In erythrocytes (RBCs), enzyme activity increased from undetectable to 20 to 30% of healthy controls (Fig. 4A) and was detectable in myeloid progenitors differentiated in vitro (CFU-GM) (550 units; normal values, 5000 ± 2000 units). In Pt2, BM ADA activity increased eightfold (from 180 to 1500 units) after gene

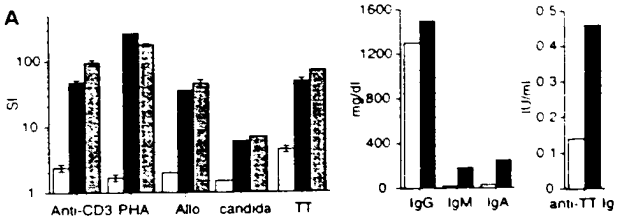

Fig. 2. Restoration of immune functions (**A**) Pt1 (Left) Proliferative responses to anti-CD3 mAb, PHA, alloantigen, and candida stimuli before (open bars) and after (solid bars) gene therapy, compared with age-matched control (gray bars). Proliferative responses to tetanus toxoid (TT) before (open bars) and after (solid bars) vaccination are compared with age-matched control (gray bars). Proliferative responses were evaluated by [^{3}H]thymidine incorporation and are represented as stimulation index [SI, ratio of counts per minute (cpm) with the stimuli over cpm with medium alone] (*13*, *25*). (Middle) Serum antibody levels before (while on IVIG) (open bars) and after (solid bars) gene therapy. (Right) Serum levels of antibody specific to TT before (open bars) and after (solid bars) vaccination with TT. Patient was vaccinated 3 months after discontinuation of IVIG. Protective values for anti-TT antibodies are >0.1 international unit/ml. (**B**) Pt2. (Left) Proliferative responses to anti-CD3, PHA, and alloantigen stimuli before (open bars) and after (solid bars) gene therapy, compared with age-matched control (gray bars). (Right) Serum immunoglobulin levels before (open bars) and after (solid bars) gene therapy.

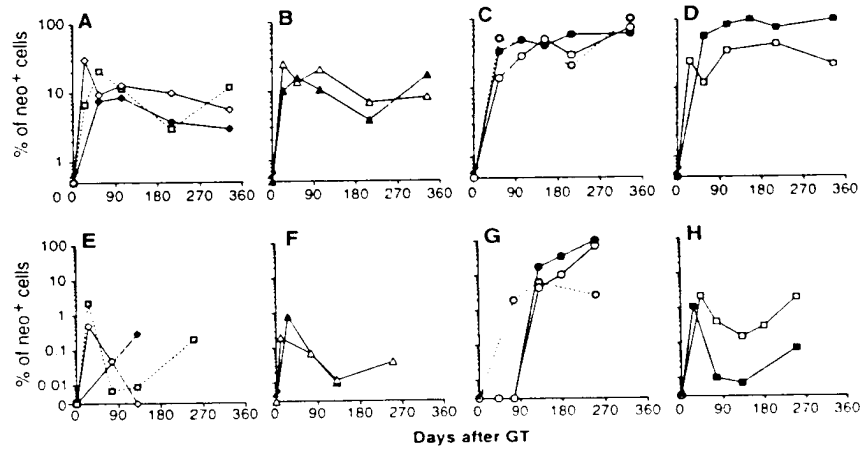

Fig. 3. Quantitative PCR analysis for vector-containing cells. Cells were sorted by flow cytometry (*13*, *26*). DNA was isolated and analyzed by real-time Q-PCR analysis (*13*) for the proportion of vector-positive (Neo^+) cells in Pt1 (**A** to **D**) and Pt2 (**E** to **H**). Graphs show percent of Neo^+ cells in the following sorted cell populations: (A and E) $CD34^+$ progenitor cells (gray squares), $CD61^+$ megakaryocytic cells (solid diamonds), and glycophorin A^+ erythroid cells (open diamonds) from the BM; (B and F) $CD15^+$ granulocytic cells from the BM (open triangles) or the PB (solid triangles); (C and G) $CD3^+$ T cells (open circles), $CD56^+/CD16^+$ NK cells (gray circles) and T-cell lines (solid circles) from the PB; (D and H) $CD19^+$ B cells from the BM (open boxes) or the PB (solid boxes).

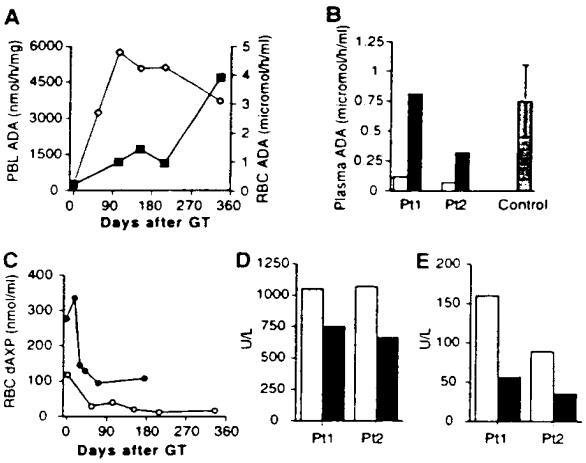

Fig. 4. Biochemical studies for ADA and purine metabolites. (**A**) Intracellular ADA activity in PBL (solid boxes) (units expressed as nmol·hour^{-1}·mg^{-1}) and in RBC (open diamonds) (units expressed as μmol·hour^{-1}·ml^{-1}) of Pt1. Normal ADA activity in PBL: 1350 ± 650 nmol hour^{-1} mg^{-1}. Normal ADA activity in RBC: 12 ± 2 μmol hour^{-1} ml^{-1}. Two and a half months before gene therapy Pt1 received an exchange transfusion of RBC, which might have reduced the initial dAXP value. (**B**) ADA activity in plasma of Pt1 and Pt2 before (open bars) and after (solid bars) gene therapy and in age-matched controls (gray bars) (units expressed as μmol of plasma per hour per ml). Normal ADA activity in plasma: 0.7 ± 0.3 units. (**C**) Concentration of dAXP purine metabolites in RBC, measured at different time points of follow-up in Pt1 (open circles) and Pt2 (solid circles), expressed as nmol/mL. Normal values: 0 nmol/ml. (**D**) Serum levels of LDH in Pt1 and Pt2 before (open bars) and after (solid bars) gene therapy (normal LDH values, 300 to 600 units/liter). (**E**) Serum levels of aspartate aminotransferase (AST) before (open bars) and after (solid bars) gene therapy (normal AST values, 2 to 60 units/liter).

therapy. ADA activity in the plasma increased in both patients after gene therapy (Fig. 4B). This increase was paralleled by a decline in RBC toxic adenine deoxyribonucleotide (dAXP) metabolites to levels equal to 10 and 40% of the initial value for Pt1 and Pt2, respectively—levels comparable to those found in patients successfully transplanted with allogeneic BM (*21*, *22*) (Fig. 4C). The amelioration of the metabolic pattern was followed by a normalization of lactate dehydrogenase (LDH) and liver enzymes usually elevated in ADA-SCID (*8*, *9*) (Fig. 4, D and E). During this follow-up, the two patients were in good clinical condition and did not experience any severe infectious episodes. Both patients are currently at home and clinically well, with normal growth and development. They live a normal life in their native countries and remain off enzyme replacement therapy.

Several explanations may account for the different levels of engraftment of transduced cells and restoration of the immune functions in these two patients. First, Pt2 received one log (one order of magnitude) lower autologous transduced CD34$^+$ cells than Pt1. Second, Pt2 was enrolled at an older age, which can be a crucial factor for HSC engraftment, as shown in BMT transplantation in SCID (*23*). An additional, and possibly more important, variable may be the degree of host BM ablation. Indeed, the pharmacologic biodistribution of busulfan might have differed in the two patients, because Pt1 received the drug intravenously and Pt2 received it orally. These results suggest that early intervention with optimal amounts of transduced HSCs and adequate conditioning are crucial factors in determining the speed and level of engraftment.

A similar gene transfer protocol was used to transduce BM CD34$^+$ cells of two SCID-X1 patients (*7*) without conditioning. In this study, gene therapy resulted in the development of T and NK corrected cells, allowing full reconstitution of T cell functions, whereas B lymphocytes and other hematopoietic cells remained mostly untransduced. The use of conditioning is most likely responsible for the improved results of our protocol, and this advantage may counteract the toxic effects and potential complications associated with low-dose busulfan.

Overall, our results prove the safety and efficacy of HSC gene therapy combined with nonmyeloablative conditioning in restoring lymphoid development and functions and in correcting the metabolic defect of ADA-SCID with complete reversal of the clinical phenotype, in the absence of enzyme replacement. These results represent a significant advance over the pioneering studies of gene therapy with PBL in ADA-SCID patients receiving PEG-ADA that showed efficient gene transfer into long-living T lymphocytes (*1*, *24*).

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Supporting Online Material

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Materials and Methods

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Systemic delivery of an adenoviral vector encoding canine factor VIII results in short-term phenotypic correction, inhibitor development, and biphasic liver toxicity in hemophilia A dogs

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Canine hemophilia A closely mimics the human disease and has been used previously in the development of factor VIII (FVIII) protein replacement products. FVIII-deficient dogs were studied to evaluate an in vivo gene therapy approach using an E1/E2a/E3-deficient adenoviral vector encoding canine FVIII. Results demonstrated a high level of expression of the canine protein and complete phenotypic correction of the coagulation defect in all 4 treated animals. However, FVIII expression was short-term, lasting 5 to 10 days following vector infusion. All 4 dogs displayed a biphasic liver toxicity, a transient drop in platelets, and development of anticanine FVIII antibody. Canine FVIII inhibitor development was transient in 2 of the 4 treated animals. These data demonstrate that systemic delivery of attenuated adenoviral vectors resulted in liver toxicity and hematologic changes. Therefore, the development of further attenuated adenoviral vectors encoding canine FVIII will be required to improve vector safety and reduce the risk of immunologic sequelae, and may allow achievement of sustained phenotypic correction of canine hemophilia A. (Blood. 2001;97: 107-113)

Introduction

Hemophilia A is a severe, X-linked bleeding disorder caused by a deficiency of blood coagulation factor VIII (FVIII). Hemophilia A has an incidence approaching 1 in 4000 males in all populations,[1] and in its severe form, is a life-threatening, crippling disease. Infusion of plasma-derived or recombinant FVIII protein in response to bleeding crises is currently the most widely accepted therapy[1] and has dramatically increased the life expectancy and quality of life for many patients with hemophilia. However, the high cost and short supply of FVIII replacement products has resulted in their availability being limited to less than 10% of the world's hemophilic population.

Gene therapy for hemophilia A would provide prophylactic expression of FVIII and correction of the coagulation defect. Considerable progress has been made recently in the development of adenoviral vector-mediated gene therapy for hemophilia A.[2,3] Potent adenoviral vectors encoding a human FVIII complementary DNA (cDNA) have been developed that mediated expression of physiologic levels of FVIII in mice,[4-7] monkeys,[8] and dogs,[9] and sustained human FVIII expression in normal[5] and hemophilic mice.[7] Treatment of hemophilic mice and dogs resulted in human FVIII expression and complete phenotypic correction, verifying the feasibility of adenoviral vector administration for the treatment of hemophilia A.[7,9-11] Expression in the hemophilic mice was sustained for at least 1 year,[7,11] whereas the duration of expression in the hemophilic dogs was short-term, limited by a rapid antibody response to the human FVIII protein.[9]

Canine hemophilia A was first described 50 years ago,[12,13] and FVIII-deficient dogs have been used to support the development of FVIII pharmaceutical products.[14-19] However, human FVIII is highly immunogenic in dogs when the protein is delivered intravenously[20] or via gene therapy.[9] In contrast, canine FVIII is significantly less immunogenic than the human protein in hemophilic dogs and most animals can be repeatedly treated with canine plasma without developing inhibitory antibodies.[21] The establishment of sustained phenotypic correction in hemophilic dogs may require the development of adenoviral vectors that encode the canine cDNA.

Recently, we isolated the canine FVIII cDNA[22] and constructed a third generation, E1/E2a/E3-deleted[23] adenoviral vector encoding canine FVIII.[11] Comparison of the FVIII expression level of the canine vector to that of an analogous vector encoding a human FVIII cDNA in hemophilic mice demonstrated expression of the canine protein at levels at least 10-fold higher than that of human FVIII.[11] Canine FVIII expression was sustained for over 1 year in the hemophilic mice.[11]

In this work, we treated 4 FVIII-deficient hemophilic dogs with the canine FVIII adenoviral vector. Two dogs received a higher vector dose (3×10^{12} particles/kg) and the other 2 dogs received a 5-fold lower vector dose (6×10^{11} particles/kg). We measured whole blood clotting time (WBCT) and cuticle bleeding time (CBT), canine FVIII plasma activity levels, liver enzymes, platelet and fibrinogen levels, and the development of canine FVIII inhibitory antibodies.

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Materials and methods

Construction of recombinant adenoviruses

Cloning of the canine FVIII cDNA and 3′ untranslated region,[22] construction of the B-domain–deleted canine FVIII cDNA, and generation of the canine FVIII-encoding recombinant adenovirus, Av3H8401, were described previously.[11] The analogous, recombinant adenovirus encoding human FVIII, Av3H8101, has been described.[7] Vector concentrations were determined by spectrophotometric analysis.[24] Titers are stated as particles per milliliter. The vector preparations were checked for the presence of replication-competent adenovirus contamination by polymerase chain reaction (PCR) directed at E1a sequences,[25] and all vector preparations contained fewer than 10 particles of E1a-containing vector per 10^8 particles.

Hemophilic dog procedures and adenoviral vector administration

The experimental animals used in this study were mixed-breed dogs from the hemophilia A colony housed at Queen's University.[13] Phlebotomy was performed from the cephalic vein. The adenoviral vector was diluted in Hanks' buffered saline solution (Life Technologies, Gaithersburg, MD) and was administered through an in-dwelling cephalic vein catheter by slow infusion at a rate of 3 mL/min. All animals were housed in facilities accredited by the Canadian Council for Animal Care and experimental procedures were approved by both the Genetic Therapy, Inc, and Queen's University Animal Care Committees.

Coagulation and FVIII-specific assays

The WBCT was measured following standard procedures. CBT was measured as described.[13,15,21] Briefly, dogs were lightly anesthetized and placed in a supine position, and the fur around the nail was clipped. The nail was then severed directly proximal to the dorsal nail groove and the time in minutes until clot formation was recorded. The one-stage FVIII coagulant analyses (based on the activated partial thromboplastin time, APTT) were performed on the dog plasma samples using the Organon Teknika Automated APTT reagent (Durham, NC) and an automated coagulometer (General Diagnostics Coag-A-Mate, Toronto, ON, Canada) following the manufacturer's instructions. Both human reference plasma (Normal Reference Plasma, Precision Biologicals, Dartmouth, NS, Canada) and canine pooled plasma were used as the controls. The limit of sensitivity of the one-stage coagulant assay using canine plasma was less than 300 mU/mL.

Biologically active canine FVIII was also measured in the dog plasma using the Coatest chromogenic bioassay (DiPharma, West Chester, OH). Coatest measures the FVIII-dependent generation of factor Xa from factor X, with 1 U defined as the amount of FVIII activity in 1 mL of pooled human plasma, 100 to 200 ng/mL.[26] Pooled human plasma (George King Bio-Medical, Overland Park, KS) was used as the FVIII activity standard to generate a standard curve. When compared to human plasma, normal canine plasma FVIII levels were 5- to 10-fold higher.[9,27,28] The Coatest assay displayed a limit of sensitivity of 7 mU/mL in the presence of canine plasma.

Anticanine FVIII inhibitory antibodies were measured using the Bethesda assay.[29] Various dilutions of test plasmas were mixed 1:1 with a normal canine plasma pool and incubated at 37°C for 2 hours and the residual FVIII coagulant activity determined with a conventional one-stage FVIII:C assay. The dilution with residual activity closest to 50% was used to calculate the inhibitor titer, in which 50% residual FVIII activity equals 1 Bethesda unit (BU) per milliliter.

Hematology assays

Fibrinogen assays and platelet counts were performed at Queen's University, using standard procedures. Fibrin split products were measured using the semiquantitative D-dimer ACCUCLOT kit (Sigma Diagnostics, St Louis, MO). To assay liver toxicity, serum was collected before and at the indicated times following vector administration, centrifuged, aliquoted, and frozen. A total of 60 μL of each serum sample was submitted to Ani-Lytics, Inc (Gaithersburg, MD) and analyzed for the presence of aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (AP), and total bilirubin levels.

Results

High-level canine FVIII expression and phenotypic correction of canine hemophilia A

The recombinant adenoviral vector, Av3H8401,[11] contains the mouse albumin promoter, an intron from the human *apolipoprotein A1* gene, a canine B-domain deleted (BDD) FVIII cDNA, and 1.5 kb of the canine 3′ untranslated region (UTR). The vector backbone was derived from adenovirus serotype 5 (Ad5) and is devoid of the E1, E2a, and E3 regions.[23] Evaluation of Av3H8401 in hemophiliac mice demonstrated expression of canine FVIII sustained for at least 1 year at levels 10-fold higher than human FVIII expression from an analogous vector.[11]

Av3H8401 was administered to 4 dogs by cephalic vein infusion (Table 1). Dogs A and B received a vector dose of 3×10^{12} particles/kg; dogs C and D received a 5-fold lower vector dose (6×10^{11} particles/kg). WBCT was measured before vector infusion and at the indicated times following treatment (Figure 1). Prior to treatment all 4 dogs displayed an abnormal WBCT of more than 15 minutes. At 1 day, the WBCT was normalized to less than 5 minutes in all 4 animals. The WBCT remained corrected for 3 to 5 days after which the WBCT returned to abnormal levels.

The CBT, an in vivo test sensitive to discrete coagulation factor

Table 1. Treatment summary of FVIII-deficient dogs

Animal	Gender	Age (mo)	Weight (kg)	Vector dose (part/kg)	Peak FVIII plasma activity (mU/mL)		FVIII antibody titer (BU)	
					APTT	Coatest	Peak	Pre/Post reinfusion
Dog A (Riley)	Male	10	11.8	3×10^{12}	18 700	58 680	1210	41/51
Dog B (JJ)	Male	10	10.0	3×10^{12}	19 900	43 591	37	1.7/2.5
Dog C (Cocoa)	Female	12	12.0	6×10^{11}	1347	1335	1.9	0/0
Dog D (Java)	Male	12	18.5	6×10^{11}	1427	1725	1.4	0/0

Four hemophilic dogs were treated with the canine FVIII-encoding adenoviral vector, Av3H8401, via cephalic vein infusion. Age and weight of the animals directly prior to vector administration is displayed. Plasma samples were analyzed for FVIII biologic activity using the one-stage FVIII coagulant assay (APTT) and the Coatest chromogenic bioassay. Peak FVIII plasma levels were obtained 2 days after vector treatment in all 4 dogs. Plasma samples collected prior to and following vector treatment were analyzed for the presence of anticanine FVIII inhibitory antibodies using the Bethesda assay. All animals displayed a Bethesda titer of 0 prior to vector treatment. Peak FVIII antibody titers were obtained 12 days after vector treatment in dogs A and B and 14 days after vector treatment in dogs C and D. Canine cryoprecipitate (20 FVIII Unit/kg) was delivered by IV administration to dogs A and B at 26 months and dogs C and D at 20 months after vector treatment. Directly prior to and 10 days following FVIII protein infusion, antibody levels were measured. Dog A received 2 infusions of cryoprecipitate (20 FVIII U/kg). Following the first infusion, antibody levels were followed for 4 weeks with no change detected. The data displayed are antibody levels following the second protein infusion.

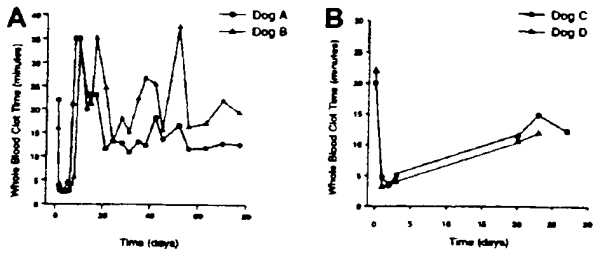

Figure 1. WBCT of vector-treated hemophilic dogs. Blood was collected from the dogs at the indicated times prior to and following vector treatment and assessed for WBCT. (A) Dogs treated with Av3H8401 at a dose of 3×10^{12} particles/kg. (B) Dogs treated with Av3H8401 at a dose of 6×10^{11} particles/kg. Pretreatment WBCT for dog A was 22 minutes; at 22 months, WBCT was 16 minutes. Pretreatment WBCT for dog B was 16 minutes; at 22 months, WBCT was 20 minutes. Pretreatment WBCT for dog C was 20 minutes; at 16 months, WBCT was 16 minutes 45 seconds. Pretreatment WBCT for dog D was 22 minutes; at 16 months, WBCT was 15 minutes 45 seconds.

deficiencies,[13] was performed before vector treatment and after vector administration. The normal range for clot formation in dogs following cuticle severing is 2 to 8 minutes.[13] For dogs C and D, CBT before vector treatment was 13 and 14 minutes, respectively, indicative of the hemophiliac phenotype. At 2 days, CBT was completely corrected at 4 minutes for both animals. CBT for dogs A and B before treatment was 11 and 12 minutes, respectively. At 6 days, dog A's CBT was abnormal at 12 minutes, and dog B displayed a CBT of 9 minutes. CBT was not performed on dogs A and B at 2 days. Normalization of the WBCT and CBT coagulation parameters demonstrated complete, albeit transient, phenotypic correction of canine hemophilia A.

Canine FVIII biologic activity was determined by analysis of plasma samples collected before and after vector administration, using the one-stage FVIII coagulant assay (based on the APTT), and the FVIII chromogenic bioassay, Coatest (Figure 2). Prior to treatment, FVIII biologic activity detected by APTT was less than 300 mU/mL, and no FVIII was detected in the plasma using the Coatest assay. Peak FVIII expression in all 4 treated dogs was detected at 2 days with both the APTT and Coatest assays. Dogs A and B displayed extremely high plasma levels of FVIII, up to 58 times normal human FVIII plasma levels[26] (Table 1). Dogs C and D displayed peak FVIII plasma levels of 1 to 2 U/mL, equivalent to normal human physiologic levels[26] (Table 1). FVIII expression persisted in dog A for 5 days, whereas dogs B, C, and D displayed FVIII expression out to 9 days (Figure 2). The FVIII expression values obtained using the APTT and Coatest assays were in good agreement; however, Coatest activity values at high FVIII expression levels were routinely 2- to 3-fold higher than those detected by the APTT assay (Figure 2). This discrepancy between assays had been noted previously when using a recombinant B domain-deleted FVIII concentrate and appears to be phospholipid dependent.[30]

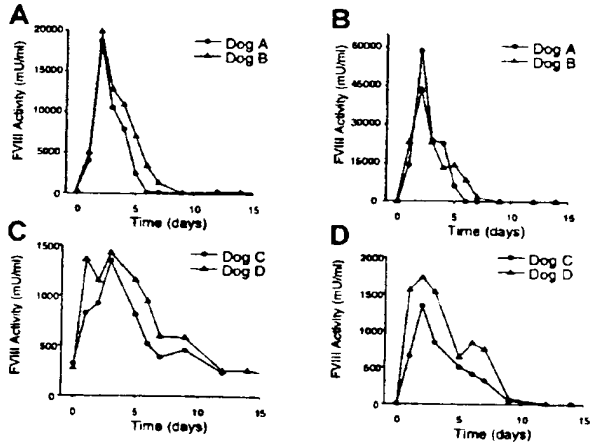

Figure 2. Plasma FVIII expression levels in treated hemophilic dogs. Plasma was collected from the dogs at the indicated times prior to and following vector treatment and assessed for FVIII biologic activity. (A) One-stage FVIII coagulant assay (APTT) plasma analyses from dogs treated with the higher vector dose (3×10^{12} particles/kg). (B) Coatest bioassay plasma analyses from dogs treated with higher vector dose (3×10^{12} particles/kg). (C) One-stage FVIII coagulant assay (APTT) plasma analyses from dogs treated with the lower vector dose (6×10^{11} particles/kg). (D) Coatest bioassay plasma analyses from dogs treated with the lower vector dose (6×10^{11} particles/kg).

Liver toxicity and inhibitor development in treated dogs

Peripheral vein administration of adenoviral vectors results in efficient liver transduction in mice,[31] dogs,[9] and monkeys.[8] We have shown previously that vector-induced hepatotoxicity resulted in short-term FVIII expression caused by the loss of vector DNA from the transduced mouse livers.[9] Therefore, the persistence of FVIII expression in the treated dogs may have been limited by the direct toxicity of the vector,[9] a cellular immune response against expressed adenoviral genes or the FVIII transgene,[32-35] or the development of FVIII-specific inhibitory antibodies.[9]

To measure hepatotoxicity in the treated dogs, serum collected before and after vector administration was analyzed for the presence of several liver enzymes: ALT (Figure 3), AST, AP, and bilirubin (data not shown). Elevation of ALT and AST indicates hepatocellular necrosis, whereas increased levels of AP and bilirubin correlate with biliary obstruction.[36] All 4 treated dogs displayed a significant increase in the ALT, AST, and AP levels. Interestingly, both animals that received the higher vector dose (dogs A and B; Figure 3A) and the lower vector dose (dogs C and D; Figure 3B) showed a similar pattern of ALT accumulation in the serum. Following an initial elevation in ALT levels 1 to 3 days after vector infusion, the ALT levels began to resolve at days 4 to 5, and then displayed a dramatic increase at days 7 to 12. However, the initial elevation in ALT levels was attenuated in the animals that received the lower vector dose, whereas the secondary elevation was similar in both groups of dogs (Figure 3). AST and AP displayed a similar accumulation pattern (data not shown). Bilirubin was detected in the serum of dog B 9 days after vector infusion at a level 2- to 3-fold higher than normal, and levels returned to normal by day 12. No accumulation of bilirubin was detected in the

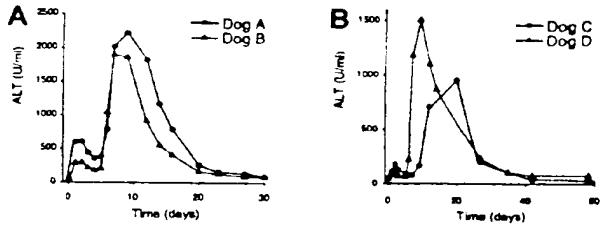

Figure 3. Plasma liver enzyme analyses. Plasma was collected from the dogs at the indicated times prior to and following vector treatment and assessed for the presence of the liver enzyme ALT. (A) ALT analyses of dogs that received the higher vector dose (3×10^{12} particles/kg). (B) ALT analyses of dogs that received the lower vector dose (6×10^{11} particles/kg). Pretreatment ALT value for dog A was 54 U/mL; at 22 months, ALT was 69 U/mL. Pretreatment ALT value for dog B was 47 U/mL; at 22 months, ALT was 55 U/mL. Pretreatment ALT value for dog C was 46 U/mL; at 16 months, ALT was 33 U/mL. Pretreatment ALT value for dog D was 44 U/mL; at 16 months, ALT was 47 U/mL.

other 3 treated dogs. These data demonstrate that vector administration to the hemophilic dogs resulted in a biphasic hepatotoxicity that resolved over the course of the study (16-22 months).

To determine if the loss of FVIII expression was due to the development of canine FVIII-specific inhibitory antibodies, Bethesda assays[29] were used to measure FVIII inhibitors in plasma samples before and after treatment. All 4 dogs displayed no detectable Bethesda titer before vector treatment (Table 1, Figure 4). Dog A developed a high-level anticanine FVIII antibody titer (109 BU) by day 6 following vector treatment, which increased rapidly to a peak of 1210 BU at days 12 and 14 (Figure 4). The inhibitor titer decreased rapidly, however, and by day 69 was reduced to 145 BU (Figure 5). At 22 months, dog A had an anticanine FVIII titer of 46 BU. The occurrence of anticanine FVIII antibodies in dog A was not unexpected because this animal was from the line of hemophilic dogs within the Queen's University colony, genetically predisposed to the development of canine FVIII-specific antibodies.[21] In contrast, dogs B, C, and D were from the hemophilic dog line that does not develop antibodies even after repeated treatment with normal canine plasma or cryoprecipitate.[21] Dog B developed an anticanine FVIII inhibitor titer of 2.4 BU by day 9, which increased to a peak of 37 BU by day 14 (Table 1, Figure 4). This inhibitor titer also decreased rapidly and by day 69 was 5.2 BU and at 22 months was 2.3 BU (Table 1, Figure 4). In contrast, the dogs that received the lower vector dose, dogs C and D, developed low-level, transient, inhibitors (1-2 BU) lasting 1 (dog D) to 4 (dog C) weeks (Figure 4). By 16 months, both dogs C and D remained inhibitor-free (Figure 4).

The effect of infusion of canine FVIII protein on the titer of FVIII inhibitory antibodies in the vector-treated dogs was evaluated. FVIII cryoprecipitate (20 U/kg) was delivered through intravenous administration to dogs A and B at 26 months and to dogs C and D at 20 months following initial vector exposure. Inhibitory antibody levels were measured directly before and 10 days after protein administration (Table 1). No significant increase in inhibitory antibody titer was detected in any animal. Furthermore, dog A received 2 infusions of FVIII cryoprecipitate (20 U/kg). Following the first infusion, antibody levels were followed for 4 weeks with no change detected (data not shown). The second protein administration was performed simultaneously with the other 3 dogs. Following the second infusion, again, no increases in antibody levels were detected (Table 1).

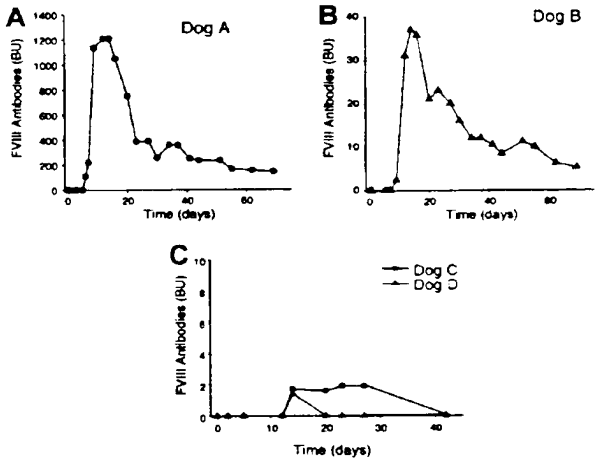

Figure 4. Anticanine FVIII inhibitory antibody development in treated dogs. Plasma was collected from the dogs at the indicated times prior to and following vector treatment and tested for the presence of FVIII inhibitory antibodies by Bethesda assay. (A) Bethesda titer of dog A. (B) Bethesda titer of dog B. (C) Bethesda titer of dogs C and D. All animals displayed a Bethesda titer of 0 prior to vector treatment. Final titer for dog A was 46.0 BU at 22 months; final titer for dog B was 2.3 BU at 22 months; final titer for dog C was 0 BU at 16 months; and final titer for dog D was 0 BU at 16 months.

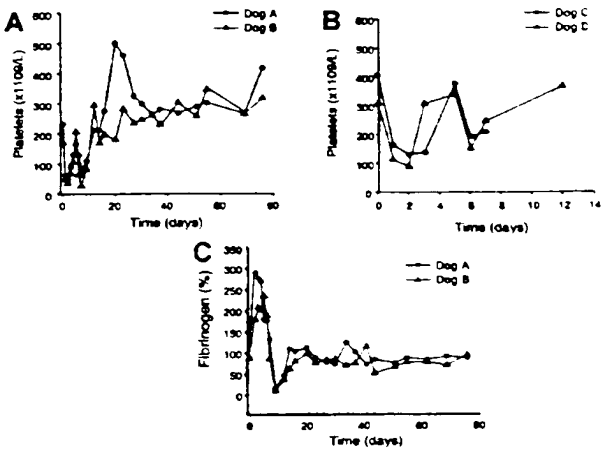

Figure 5. Platelet counts and fibrinogen levels of treated dogs. Blood was collected from the dogs at the indicated times prior to and following vector treatment and platelet and fibrinogen levels were measured. (A) Platelet counts in the dogs treated with the higher vector dose (3×10^{12} particles/kg). (B) Platelet counts in the dogs treated with the lower vector dose (6×10^{11} particles/kg). (C) Fibrinogen levels in the dogs treated with the higher vector dose. Pretreatment platelet counts for dog A were 231 (× 1109/L); 22-month counts were 276 (× 1109/L). Pretreatment platelet counts for dog B were 169 (× 1109/L); 22-month counts were 240 (× 1109/L). Pretreatment platelet counts for dog C were 405 (× 1109/L); 16-month counts were 185 (× 1109/L). Pretreatment platelet counts for dog D were 312 (× 1109/L); 16-month counts were 168 (× 1109/L).

Platelet counts, fibrinogen levels, and fibrin split products in treated dogs

Platelet and fibrinogen levels in the 2 dogs treated with the higher vector dose, dogs A and B, were followed for 76 days after vector administration (Figure 5A,C). Two days after vector treatment, the platelet levels in both dogs dropped approximately 80%. Levels remained low until day 12, after which the platelet levels increased to levels up to 2-fold higher than the pretreatment values. In contrast, fibrinogen levels increased 1.5- to 3-fold 2 days after vector treatment and remained elevated until day 7 (Figure 5C), likely representing an acute phase response to the vector delivery and initial hepatotoxicity. At day 9, however, fibrinogen levels dropped sharply coincident with the second phase of hepatotoxicity (Figure 3A). By days 14 to 16, fibrinogen levels were completely normalized to pretreatment values and remained normal to 22 months. Analysis of the day 9 plasma from dogs A and B revealed the presence of fibrin split products, suggesting the occurrence of disseminated intravascular coagulation (DIC). Platelet counts in the 2 dogs receiving the lower vector dose, dogs C and D, also showed an initial 70% decline 1 to 2 days following vector treatment, which resolved by 12 days (Figure 5B). Fibrinogen measurements were not performed with dogs C and D. Fibrin split products were not detected in day 5 plasma samples from dogs C and D.

Discussion

This work represents the first report of in vivo gene therapy of FVIII-deficient dogs using an adenoviral vector-encoding canine FVIII. Expression of the canine protein in hemophilic dogs would be analogous to treating humans with hemophilia with vectors encoding the human protein. Previous work demonstrated that expression of human FVIII in hemophilic dogs resulted in the development of a strong, antihuman FVIII antibody response and short-term FVIII expression.[9] Because canine FVIII is significantly less immunogenic in hemophilic dogs than the human protein,[20,21] we reasoned that sustained phenotypic correction of canine hemophilia A would require the expression of canine FVIII. In this work, we have achieved an extremely high level of expression of canine FVIII in the hemophilic dogs and complete phenotypic correction of the bleeding disorder. However, FVIII expression was short-term, lasting less than 2 weeks. All treated animals developed severe liver toxicity that resolved over the course of the study. All 4 dogs developed canine FVIII inhibitory antibodies, which in one animal, genetically predisposed to inhibitor development, reached a peak of 1210 BU; the 2 animals treated with the lower vector dose developed low-level, transient inhibitory antibodies lasting 1 to 4 weeks.

The hepatotoxicity observed in the treated dogs displayed a biphasic pattern. The first phase was dose-dependent, whereas the second phase was dose-independent. Dogs that received the higher vector dose initially showed a 6- to 11-fold increase in ALT levels, whereas dogs that received the 5-fold lower vector dose initially showed a 2- to 4-fold increase in ALT levels. In all animals, the ALT levels began to normalize 3 days after treatment but by 7 days had increased sharply to extremely high levels. The initial, dose-dependent hepatotoxicity may have been caused by the direct toxicity of the vector to the transduced hepatocytes, similar to what had been observed previously in FVIII adenoviral vector-treated mice.[5]

The secondary, dose-independent rise in liver enzyme levels at 7 days may be best explained by the development of a cellular immune response directed against transduced hepatocytes expressing adenoviral backbone genes or the canine FVIII transgene or both. Previous studies, using mouse models, have demonstrated that adenoviral vectors or immunogenic transgenes may be associated with cytotoxic T lymphocyte-mediated elimination of transduced cells and, thereby, the loss of gene expression.[32,33,35,37-39] In many cases, the response to the transgene or viral backbone gene expression was influenced by the mouse strain used in the study.[38,40] Therefore, the use of a large, out-bred animal model such as the hemophilic dogs may complicate the dissection of the immune components involved in adenoviral vector-mediated gene therapy of canine hemophilia A. However, the further attenuation of viral backbone gene expression, by the generation of gutless adenoviral vectors from which all viral backbone genes have been removed,[41] will reduce the response to viral gene products and may reduce vector toxicity.[42-45]

Alternatively, the hepatotoxicity observed in the treated dogs may have been caused by the expression of extremely high levels of canine FVIII. A high level of FVIII protein expression is known to be toxic to cultured rodent cells in vitro (unpublished observations). However, this possibility seems unlikely because dogs C and D, animals that received the lower vector dose and displayed significant liver toxicity, expressed canine FVIII at low levels, 1 to 2 U/mL, which is 5- to 10-fold lower than the FVIII plasma levels measured in normal dogs (5-10 U/mL).[9,27,28]

Sustained canine FVIII expression in the vector-treated dogs was also limited by the development of canine FVIII inhibitory antibodies. The 2 dogs that received the higher vector dose displayed the higher initial elevation in liver enzyme levels and rapidly developed high titers of canine FVIII inhibitor. The occurrence of canine FVIII inhibitory antibodies in dog A was not unexpected because this animal was derived from the line of hemophiliac dogs within the colony predisposed to the development of canine FVIII-specific antibodies.[21] This propensity to inhibitor development is genetically based and independent of the FVIII mutation.[21] However, the mechanism of inhibitor development remains uncertain and is the subject of continued research in the laboratory. In contrast, dogs B, C, and D, derived from the hemophiliac dog line that does not routinely develop canine FVIII-specific antibodies,[21] also developed inhibitors. Of these animals, dog B, who received the higher vector dose, showed the highest inhibitor level (peak of 37 BU), whereas dogs C and D showed low (1-2 BU) antibody levels persisting for less than 1 month. These observations suggest that a higher vector dose and, perhaps, more severe vector-induced hepatotoxicity were responsible for inhibitor development in these animals. It is possible, therefore, that vector-mediated liver toxicity resulted in the hyperreactivity of the immune system to canine FVIII, potentially caused by the incomplete or incorrect processing of the canine FVIII protein in the transduced hepatocytes. This hypothesis suggests that elimination of the liver toxicity may also limit the development of canine FVIII-specific inhibitory antibodies in hemophilic dogs that are not genetically prone to inhibitor generation. Indeed, FVIII-deficient mice treated with an analogous vector encoding human FVIII display minimal hepatotoxicity[7,11] and do not develop an immune response directed toward human FVIII.[7] In contrast, a recent report has demonstrated sustained expression of human FVIII in some hemophilic mice treated with an adenoviral vector lacking all viral genes.[43] Although no hepatotoxicity was observed with this attenuated vector, 13 of 16 treated mice developed antihuman FVIII antibodies, which resulted in short-term expression of the human protein.[43] It will be interesting to test a similar, highly attenuated vector in the FVIII-deficient canine model.

Interestingly, infusion of canine FVIII cryoprecipitate in the vector-treated dogs had no effect on the titer of inhibitory antibodies. Furthermore, dog A, the animal predisposed to the development of canine FVIII inhibitory antibodies and the animal with the highest inhibitor titer, was treated with canine cryoprecipitate twice following vector delivery. No change in inhibitor titer was detected at any time point evaluated. This observation was unexpected because infusion of canine FVIII cryoprecipitate in animals with existing inhibitor titers induced by protein infusion consistently resulted in an anamnestic response.[21] These data suggest a difference between vector-induced inhibitory antibody development and FVIII protein-induced antibody development. Further studies investigating the immunogenicity of gene therapy versus protein replacement treatment are in progress.

We found a treatment-dependent, transient decrease in mean platelet counts in all 4 treated dogs. Additionally, transient fluctuations in fibrinogen levels, peaking 3-fold higher than baseline, and then dropping to 20% of pretreatment levels, were also observed. A similar pattern of fibrinogen fluctuation was observed in monkeys treated with a first-generation adenoviral vector encoding human factor IX.[46] However, none of the dogs developed symptomatic bleeding. The 2 animals that received the higher vector dose

showed an 80% to 90% decrease in platelets, whereas the 2 animals that received the lower vector dose showed a 70% decrease in platelets. Transient thrombocytopenia caused by systemic delivery of adenoviral vectors has been described previously in mice,[47] rabbits,[47] and monkeys,[8] although its cause remains elusive. The first and most worrying hypothesis is that the drop in platelets was due to DIC. This mechanism is consistent with the transient decrease in fibrinogen levels observed on days 9 to 12 in the higher-dosed dogs and the presence of fibrin split products in the day 9 plasma samples. However, fibrin split products were not detected in the lower-dosed dogs, and none of the dogs developed symptomatic evidence of a consumptive coagulopathy. A second explanation for this transient hypofibrinogenemia may have been causally related to the second phase of hepatotoxicity. Additionally, there was no evidence of DIC in the rabbit[47] and nonhuman primate[8] models of vector-mediated thrombocytopenia. Another explanation for the decrease in platelet levels is that the vector bound to platelets resulting in sequestration in the spleen or destruction via complement activation. However, in vitro evaluation in the rabbit model yielded no evidence to support complement-mediated platelet destruction.[47] Furthermore, vector exposure did not cause platelet aggregation, loss of reactivity, or changes in morphology.[47] Two other possibilities remain largely unexplored. First, adenoviral vectors could activate endothelial cells resulting in platelet adsorption. Indeed, Channon and coworkers[48] have demonstrated that vector infusion activates endothelial cells in a rabbit artery model. Finally, vector infusion could result in a transient decrease in platelet production by suppressing megakaryocyte maturation. Megakaryocyte dysfunction has been associated with a variety of viral infections.[49] A better understanding of the mechanism of platelet loss will be necessary to determine the suitability of adenoviral vectors for the treatment of hemophilia as well as other diseases.

In this work, effective, albeit transient, correction of canine hemophilia A was achieved by systemic delivery of an adenoviral vector encoding the canine FVIII cDNA. Furthermore, important hepatotoxic and hematologic consequences to systemic delivery of adenoviral vectors were observed. The development of further attenuated vectors designed to eliminate vector-mediated toxicity and reduce the immune response to transduced cells[41] may be necessary to enable long-term FVIII expression. The achievement of sustained phenotypic correction of the coagulation defect in a clinically relevant large animal model will provide a crucial step in the verification of the feasibility of adenoviral vector-mediated gene therapy for hemophilia A.

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Factors influencing the development of an anti–factor IX (FIX) immune response following administration of adeno-associated virus–FIX

Ying Ge, Sandra Powell, Melinda Van Roey, and James G. McArthur

The present study sought to determine the impact of the route of administration of an adeno-associated virus (AAV) vector encoding human factor IX (hFIX) on the induction of an immune response against the vector and its xenogenic transgene product, hFIX. Increasing doses of AAV-hFIX were administered by different routes to C57Bl/6 mice, which typically demonstrate significant immune tolerance to hFIX. The route of delivery had a profound impact on serum hFIX levels as well as the induction of an anti-hFIX humoral immune response. At all dose levels tested, delivery of AAV-hFIX by an intramuscular (IM) route induced an antibody response against the human FIX protein and no hFIX was detected in the serum of animals even at doses of 2×10^{11} DNA viral particles (vp) of AAV-hFIX. This was in stark contrast to the mice that received AAV-hFIX by intraportal vein (IPV) administration. No anti-hFIX inhibitors were observed in any of these mice and therapeutic levels of hFIX were detected in the serum of all mice that received doses of 2×10^{10} vp AAV-hFIX and higher. When pre-existing neutralizing immunity to AAV was established in mice, AAV-hFIX administration by either the IM or IPV routes did not result in detectable serum hFIX. Although hFIX expression was not observed in mice with pre-existing neutralizing immunity to AAV, an anti-hFIX response was induced in all of the animals that received AAV-hFIX by the IM route. This was not observed in the preimmune mice that received AAV-hFIX by IPV administration. These results suggest that the threshold of inducing an immune response against a secreted transgene product, in this case the xenoprotein hFIX, is lower when the vector is administered by the IM route even in animals with pre-existing immunity to AAV. (Blood. 2001;97:3733-3737)

Introduction

A concern for those seeking to use gene therapy to correct diseases stemming from genetic deficiencies has been whether the expression of the product of a missing gene will result in an immune response against the "foreign" gene product. This is a complex issue where contributions of the vector, the transgene, and the patients' immunologic background need to be considered. The area that has occupied the greatest amount of time is the immunogenicity of the viral vectors that are used to deliver potentially therapeutic transgenes. Many studies have shown that the immunogenicity of the proteins encoded by endogenous viral genes can stimulate not only the destruction of the transduced cell, but may also lead to the cross-priming of an immune response against the transgene product itself. Recombinant adeno-associated virus (rAAV) vectors are thought to be well suited for gene replacement therapy because they are deleted of all wild-type *AAV* genes. In addition, AAV serotype 2 vectors are inefficient at transducing potent antigen-presenting dendritic cells (DCs).[1] These features greatly reduce the immunogenicity of AAV vectors.

Despite these advantages of AAV vectors, the immune interaction of the host with the transgene and vector is more complex than can be explained by the presence or absence of viral genes. For instance, AAV vectors expressing secreted transgenes, such as ovalbumin (OVA) and factor IX (FIX), have been shown to elicit immune responses against the transgene. Administration of AAV vectors expressing OVA into C57Bl/6 mice by intramuscular (IM) and intravenous (IV) routes induced anti-OVA antibodies.[2] IM administration of AAV vector encoding human FIX (hFIX) into C57Bl/6 mice also induced a humoral response directed against the transgene product.[3-5] In contrast with these findings, it has been demonstrated that administration of AAV-hFIX by an intravascular route does not induce an anti-hFIX humoral immune response.[6-8] Beyond the make-up of the gene therapy vector and the nature of the transgene, the dose of the gene therapy vector and the site of administration of the vector may contribute significantly to the immune response against the transgene product.

To explore these issues we examined the impact of the dose of vector on the induction of an anti-hFIX humoral immune response following IM or intraportal vein (IPV) delivery of AAV-hFIX. We also explored the implications of a pre-existing anti-AAV immune response on AAV-mediated hFIX expression. IM administration of AAV-hFIX vector generated a robust anti-hFIX immune response. This immune response against the transgene blocked hFIX expression in the serum of these mice. In contrast, intravascular administration of AAV-hFIX did not induce anti-hFIX antibodies and significant levels of hFIX were detected in all animals receiving at least 2×10^{10} DNA viral particles (vp) of vector. In addition, we observed that an anti-AAV immune response, generated with an AAV vector encoding an unrelated transgene, blocked AAV-hFIX transduction by both IM and IPV routes. However, pre-existing humoral immunity to AAV did not prevent the induction of an anti-hFIX immune response in mice receiving AAV-hFIX by an IM route. These results indicate that IPV administration of AAV-hFIX is superior to IM administration.

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Materials and methods

Construction and production of AAV vectors

The AAV vectors expressing green fluorescent protein,[9] β-galactosidase,[10] and hFIX were constructed and generated as described previously.[7] The hFIX complementary DNA (cDNA) was expressed from a chimeric cytomegalovirus-Moloney murine leukemia virus (CMV-MMLV) promoter-enhancer coupled to the MLV intervening sequence (IVS). This hFIX expression cassette used the bovine growth hormone polyadenylation signal sequence. AAV titers were determined by dot blot analysis.

Assessment of AAV readministration in mice

The 8-week-old C57BL/6, NIH-3, and Balb/C mice were purchased from Taconic (Germantown, NY). Mice were immunized with various doses of AAV-LacZ IV, and monitored weekly for neutralizing antibodies using serum obtained by retro-orbital bleed every 2 weeks and analyzed for hFIX expression as described below.

Detection of serum hFIX by enzyme-linked immunosorbent assay

Microtiter plates were coated with 100 μL/well of a solution containing 2 mg/mL monoclonal anti-hFIX (Boehringer Mannheim, Indianapolis, IN) diluted in 0.1 M carbonate, pH 9.6. Plates were incubated overnight at 4°C and then washed and blocked for 2 hours at room temperature with 100 μL/well of 1% (wt/vol) nonfat milk in borate-buffered saline (BBM). Test samples (50 μL/well) diluted in BBM (1:5) incubated 2 hours at room temperature and 50 μL/well of a 1:100 dilution of horseradish peroxidase (HRP)–conjugated goat antihuman factor IX antibody (Affinity Biologicals, Hamilton, ON, Canada) was added and incubated for 1.5 hours at room temperature. Color was developed for 25 minutes at room temperature with 50 μL of 1 mg/mL p-nitrophenyl phosphate in 34 mm citric acid, 67 mm dibasic sodium phosphate, 0.1% hydrogen peroxide, (vol/vol), pH 5.0 buffer. Color development was stopped with 50 μL/well 2 M sulfuric acid and the absorbance measured at 490 nm.

Determination of AAV virus-specific serum by enzyme-linked immunosorbent assay

The 96-well MaxiSorp flat surface Nunc-Immuno plates were coated with 5×10^9 vp AAV in 100μL/well 0.1 M carbonate, pH 9.6, incubated overnight at 4°C, washed, and blocked with 100μL 1% bovine serum albumin/phosphate-buffered saline (BSA/PBS; incubated 2 hours at room temperature). One hundred microliters of the mouse test serum at 1:100 was added to each well and washed, and 100 μL 1:5000 rabbit antimouse IgG antibody in 0.01 M Tris-HCl, 0.25 M NaOH, pH 8.0, was added for 2 hours at room temperature. Then 100 μl/well of AKP substrate solution (Bio-Rad, Hercules, CA) was added to each well and the color allowed to develop for half-hour before stopping reactions with 50 μL 0.4 M NaOH. The plates were read at 405 nm. A positive read as more than 0.15 OD_{405}.

Anti-hFIX assay

The 96-well enzyme-linked immunosorbent assay (ELISA) plates were incubated overnight at 4°C with 2 μg/mL Bene-FIX protein in 0.1 M carbonate, pH 9.6, and then washed 5 times in wash buffer. The plate was then blocked with 89 mM boric acid, 90 mM NaCl, 1% (wt/vol) BBM and washed 3 times. Serum samples (50 μL) diluted in BBM were added to each well and the plate incubated 2 hours at room temperature. The plates were washed 3 times before adding 50 μL/well goat antimouse IgG-HRP (1:5000 dilution in BBM) and incubating plate 2 hours at room temperature. The plates were washed with 89 mM boric acid, 90 mM NaCl, pH 8.3, and 50 μL developing solution (o-phenylenediamine dihydrochloride (Sigma, St Louis, MO, P9187) in 10 mL substrate buffer; 10 μL hydrogen peroxide (Sigma, H1009) was added to each well and incubated in the dark for 30 minutes at room temperature. Reactions were stopped with 50 μL 0.4 M NaOH and the plates read at 490 nm.

Results

Immune implications of route of administration of rAAV

We sought to explore the effect of the route of administration of rAAV and dose of vector on the production of a secreted transgene product, hFIX. Increasing doses of 2×10^9 vp, 2×10^{10} vp, and 2×10^{11} vp of an AAV vector encoding hFIX were administered to C57Bl/6 mice by either direct IM administration or into the liver by way of a cannula introduced into the portal vein. Serum samples were taken from the animals at various time points following vector administration and the levels of hFIX determined by ELISA (Figure 1, top panel). The levels of hFIX detected in the serum of the mice are shown at various time points following vector administration. Expression of hFIX was not observed in mice that received AAV-hFIX by the IM route. Conversely, hFIX was detected in the serum of mice that received either 2×10^{10} vp or 2×10^{11} vp AAV-hFIX by the IPV route.

When the mouse sera were tested for the presence of anti-hFIX antibodies, we observed that high titer anti-hFIX antibody could be detected in all mice that received AAV-hFIX by the IM route (Figure 1, lower panel). In addition to being high titer, the anti-hFIX antibody responses were stable and persisted over the course of the experiment (72 days). Anti-mFIX cross-reacting antibodies did not appear to develop because there was no perturbation in serum mFIX levels or in clotting times with retro-orbital bleedings (data not shown). This immune response to hFIX following IM administration of AAV-hFIX was observed in other strains of mice including Balb/C mice (Figure 2, left panel). To examine if the anti-hFIX antibodies might be responsible for the absence of serum hFIX expression, the experiment was repeated in immunodeficient NIH-3 mice. hFIX could be readily detected in the serum in NIH-3 mice indicating that the immune response against hFIX was responsible for the absence of hFIX in the sera of mice following AAV-hFIX administered IM. IM administration of AAV-hFIX was more prone to inducing a humoral response than the intravascular IPV route.

Impact of pre-existing immunity on rAAV administration

The previous experiment was performed in AAV naive animals. Clearly, most gene therapy patients are likely to have been exposed to AAV in their lifetimes. Indeed, several previous studies have shown that more than 90% of normal blood donors tested displayed anti-AAV antibodies and between 18% and 52% of those individuals possessed neutralizing antibodies to serotype 2–derived AAV vectors.[6,11] We have previously shown that this anti-AAV immunity can be reproduced in mice by administering rAAV by an IV route and that preimmune mice are refractory to AAV-mediated gene transfer by a vascular route.[6]

These studies were expanded to examine whether rAAV-hFIX administered by a nonvascular route, specifically, IM, can circumvent pre-existing anti-AAV humoral immunity. C57Bl/6 mice were immunized by tail vein injection with 5×10^{10} vp AAV encoding lacZ. Thirty-two days after vector administration, sera from the mice were positive for the presence of anti-AAV antibody (Figure 3, top panel). As previously observed, the titer of these antibodies varied significantly from mouse to mouse. Also as previously observed, no hFIX expression could be detected after administration of 4×10^{11} vp AAV-hFIX vector by the IPV route into animals

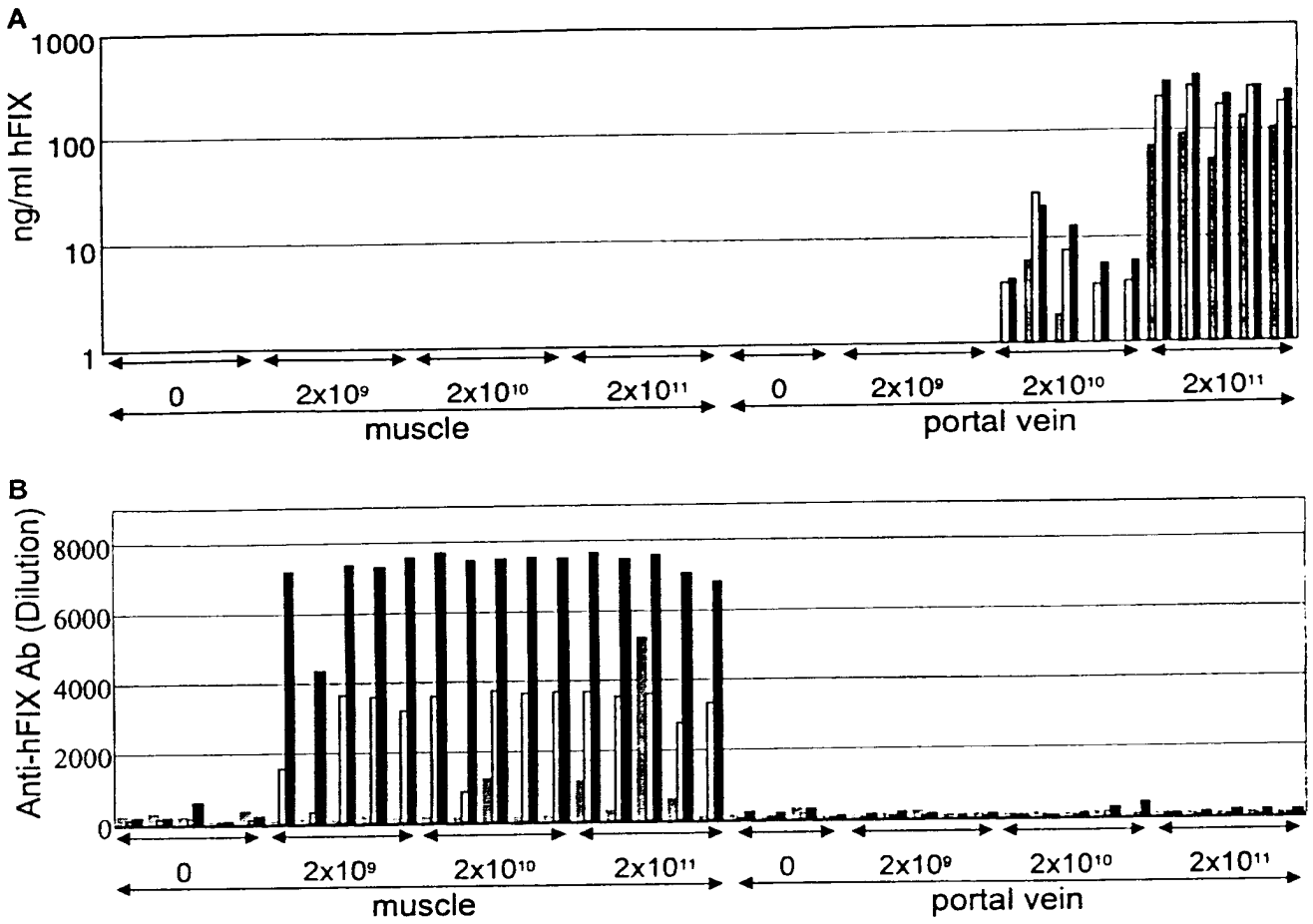

Figure 1. Impact of route of administration of AAV-hFIX on hFIX expression. Increasing doses, 2×10^9 vp, 2×10^{10} vp, and 2×10^{11} vp of AAV-hFIX were administered to C57Bl/6 mice via cannulization of the portal vein or by direct injection into the quadriceps muscle. Sera were harvested from the animals 11 (gray bars), 25 (white bars), and 41 (black bars) days after vector administration and analyzed for the presence of hFIX (upper panels) and anti-hFIX antibodies (bottom panels)

that possessed a high anti-AAV antibody titer (Figure 3, middle panels nos. 6, 7, 8, 10). The one mouse of the 5 IPV AAV-hFIX mice that did demonstrate significant levels of hFIX in the sera (Figure 3, middle panels no. 9) manifested a low anti-AAV antibody titer at the time of AAV-FIX administration (Figure 3, upper panels no. 9). A second group of 5 AAV-immune mice was transduced with 1.5×10^{11} vp AAV-hFIX vector by the IM route. Again, hFIX could not be detected in the sera of any of these mice (Figure 3, middle panels nos. 1-5).

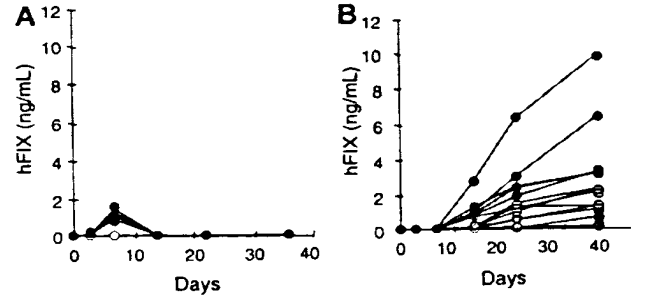

Figure 2. Immune response limits hFIX expression following IM administration of AAV-hFIX. Increasing doses, 1.2×10^{10} vp (gray circles), 6×10^{10} vp (stippled circles), and 3×10^{11} vp (solid circles) of AAV-hFIX, were administered to Balb/c (left panel) and NIH-3 (right panel) mice by direct administration into the quadriceps muscle. Sera were harvested from the animals at various time points following vector administration and analyzed for the presence of hFIX

The sera of the IM and IPV AAV-hFIX mice were next tested for the presence of anti-hFIX antibody. None of the IPV AAV-hFIX mice manifested any anti-hFIX antibody. Surprisingly, high anti-hFIX antibody titers were detected in the sera of all of the IM AAV-hFIX mice. This indicates that low levels of hFIX expressed in the IM AAV-hFIX mice were sufficient to prime an anti-hFIX antibody response but insufficient to be detected in the FIX ELISA. The hFIX ELISA can detect levels as low as 0.1 to 0.2 ng hFIX/mL mouse sera (L. Tsui, data not shown). This further suggests that IM administration of AAV-hFIX sensitizes the immune system to respond to hFIX to a far greater extent than vascular administration of AAV-hFIX.

Discussion

We sought to determine the impact of the route of administration and dose of AAV-hFIX on the expression of hFIX in the sera of immunocompetent mice. Two different routes of vector administration were chosen to target 2 tissues for which rAAV has been shown to demonstrate a tropism: muscle and liver. We observed that administration of vector via the portal vein produced higher levels of hFIX than administration of an equal dose of vector to the muscle. All of the mice that received vector by the IM route

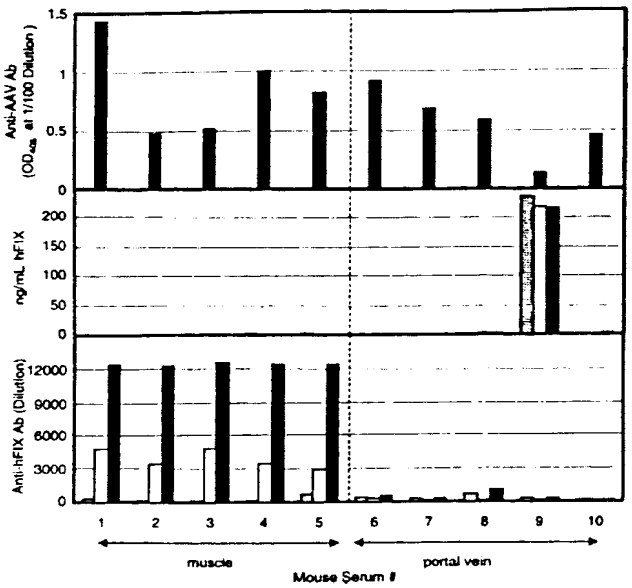

Figure 3. Anti-AAV immunity limits hFIX expression. C57Bl/6 mice were preimmunized to AAV by administering 5×10^{10} vp of AAV-lacZ by tail vein. Levels of anti-AAV antibodies were tested 1 month following vector administration (top panel). Then, 4×10^{11} vp of AAV-hFIX was administered to AAV-immune mice into the portal vein by way of a cannula or by direct administration into the quadriceps muscle. Sera were harvested from the animals 11 (gray bars), 25 (white bars), and 42 (black bars) days after AAV-hFIX administration and analyzed for the presence of hFIX (middle panels) and anti-hFIX antibodies (bottom panels).

generated a humoral immune response against the transgene despite expressing barely detectable amounts of FIX in the blood. This is to be contrasted with the observation that none of the mice that received AAV-hFIX vector by the vascular route generated an anti-hFIX antibody response. The target tissue, more so than the levels of protein expression, appeared to direct the nature of the immune response to the xenoprotein. It had been shown previously that administration of AAV encoding OVA, by either a vascular or IM route, induced anti-OVA antibodies.[2] These studies, like ours, were performed in C57Bl/6 mice with similar doses of virus. An obvious difference, however, was the transgene used in the 2 studies. We chose to conduct this study in C57Bl/6 mice because they demonstrate significant tolerance to the hFIX protein.[12] This does not appear to be the case with OVA, which induced both humoral and T-cell–mediated response by a variety of routes following *AAV* gene transfer.[2]

C57Bl/6 mice allow the stable production of hFIX in mice following intraportal administration of AAV-hFIX.[7] In long-term studies, hFIX can be observed in these AAV-hFIX mice for well over a year despite the use of a xeno-FIX protein (Brian Donahue, unpublished data, March 1997). This raises the question of whether the IM expression of AAV-FIX administration is more immunogenic than intrahepatic expression of AAV-FIX. Unfortunately, the available data to date in humans and dogs are limited due to the relatively few test subjects and variability in the doses administered. IM administration of 2×10^{11}vp/kg AAV-hFIX into 3 hemophiliac patients did not result in the induction of any detectable anti-hFIX antibodies.[13] Conversely, IM delivery of a higher dose of AAV vector encoding the canine *FIX* gene in hemophilic B dogs, induced anti-cFIX antibodies in 2 of 4 dogs that received over 10^{12} vp/kg.[3] In a separate study using hemophilic dogs, anti-cFIX antibodies were not observed in the 2 dogs that received AAV-cFIX vector by the intraportal route.[8] It is difficult to draw any firm conclusions from the 2 dog studies because the promoters driving cFIX expression in the 2 vectors differed and the numbers of animals was small. From these data we can conclude that there is a discrepancy between the IM data in normal mice with a xenogenic hFIX, the hemophiliac dog IM data with a homologous cFIX, and the clinical IM data with a homologous hFIX. It is possible that these data reflect the natural differences in the relative immune sensitivity of these different hosts to the IM presentation of FIX.

We have previously shown that a pre-existing anti-AAV neutralizing response can block AAV transduction of the liver following vascular delivery of vector.[6] It has been suggested that IM delivery of vector may allow the circumventing of the anti-AAV humoral response. We observed, however, that a pre-existing high-titer anti-AAV antibody response significantly blocked AAV-hFIX transduction of both the liver and muscle and blocked hFIX expression in the blood following either IM or hepatic administration of AAV-hFIX. Although hFIX expression was not detected in the sera of the IM AAV-hFIX mice, all of these mice demonstrated a robust anti-hFIX humoral immune response in these AAV immune mice following administration of the AAV-hFIX vector. This suggests that these mice must have expressed low levels of hFIX at some point to prime this immune response. Because these anti-hFIX immune responses persisted for over 148 days, it is also likely that there was continued low level hFIX expression.

The portal vein has access to approximately two thirds of the liver, which is a normal site of expression of FIX.[14-16] IM delivery has the practical benefit of ease of access clinically; however, it does not normally express and secrete FIX. Indeed, the muscle is generally not thought of as a secretory tissue for proteins under normal physiologic conditions. This difference may account for the observed differences in protein expression. How then could the differences in these organs' physiology produce the profound differences in the priming of an anti-hFIX immune response? It has been suggested that muscle expression of FIX protein results in binding of FIX to the muscle.[4] This inappropriate presentation of the protein might render the FIX more immunogenic. Cross-priming of immune response can be accomplished by the transfer of both major histocompatibility complex (MHC) class I and class II restricted antigen from nonantigen-presenting cells to professional antigen-presenting cells such as DCs.[1] This pathway of loading DCs with hFIX may account for the increased immunogenicity of the IM route of administration of AAV-hFIX. Limiting gene expression to the liver through the route of delivery may produce a more "physiologic" expression of the transgene. This might also be accomplished with the use of a tissue-specific promoter. Consistent with this suggestion is the observation that adenoviral vectors expressing the α_1-antitrypsin protein from tissue ubiquitous promoters are more prone to prime an immune response to this secreted transgene than vectors using a liver-specific promoter.[17]

As gene therapeutics encoding secreted proteins are advanced for a variety of diseases, a better understanding of the implications of the route and dose of vector administered is developing. AAV vectors have many valuable qualities as gene therapy vectors including the absence of any viral genes, the ability to integrate into the host cells, and the absence of viral-mediated pathology. However, most humans have been exposed to wild-type AAV and, in fact, very often the same AAV serotype 2 commonly used in gene

therapy vectors. As such, it is critical to examine the interactions of the gene therapeutic and its transgene product in both AAV-naive and AAV-immune animals. These issues become all the more important as gene therapy is applied in the treatment of diseases, such as hemophilia, where the consequences for inducing an immune response to the therapeutic transgene are severe. Our current study indicates that FIX expression following vascular delivery of AAV-hFIX is significantly less immunogenic than IM delivery in both AAV naive and AAV immune mice and that this has a profound impact on hFIX expression.

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SUSTAINED CORRECTION OF X-LINKED SEVERE COMBINED IMMUNODEFICIENCY BY EX VIVO GENE THERAPY

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ABSTRACT

Background X-linked severe combined immunodeficiency due to a mutation in the gene encoding the common γ (γc) chain is a lethal condition that can be cured by allogeneic stem-cell transplantation. We investigated whether infusion of autologous hematopoietic stem cells that had been transduced in vitro with the *γc* gene can restore the immune system in patients with severe combined immunodeficiency.

Methods CD34+ bone marrow cells from five boys with X-linked severe combined immunodeficiency were transduced ex vivo with the use of a defective retroviral vector. Integration and expression of the *γc* transgene and development of lymphocyte subgroups and their functions were sequentially analyzed over a period of up to 2.5 years after gene transfer.

Results No adverse effects resulted from the procedure. Transduced T cells and natural killer cells appeared in the blood of four of the five patients within four months. The numbers and phenotypes of T cells, the repertoire of T-cell receptors, and the in vitro proliferative responses of T cells to several antigens after immunization were nearly normal up to two years after treatment. Thymopoiesis was documented by the presence of naive T cells and T-cell antigen-receptor episomes and the development of a normal-sized thymus gland. The frequency of transduced B cells was low, but serum immunoglobulin levels and antibody production after immunization were sufficient to avoid the need for intravenous immunoglobulin. Correction of the immunodeficiency eradicated established infections and allowed patients to have a normal life.

Conclusions Ex vivo gene therapy with *γc* can safely correct the immune deficiency of patients with X-linked severe combined immunodeficiency. (N Engl J Med 2002;346:1185-93.)

DEFICIENCY of the common γ (γc) chain, an X-linked disorder, causes the most frequent form of severe combined immunodeficiency disease.[1,2] The γc chain is an essential component of five cytokine receptors, all of which are necessary for the development of T cells and natural killer cells. Without the γc chain, there is a complete absence of mature T and natural killer cells, whereas B cells are usually present in normal or increased numbers. Severe combined immunodeficiency is fatal during the first year of life because of severe, recurrent infections, unless transplantation of hematopoietic stem cells restores T-cell function.[3,4] The survival rate after transplantation of HLA-identical hematopoietic stem cells is more than 90 percent, whereas with haploidentical stem cells it is 70 to 78 percent.[3,4] In most patients, deficient B-cell function persists after transplantation and requires lifelong immune-globulin–replacement therapy.[3,5] Some patients also have persistent deficiencies of T-cell function after stem-cell transplantation.[4,6] Assessment of an al-

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ternative therapy based on the ex vivo transfer of the γc gene into autologous hematopoietic precursor cells was therefore warranted. In a preliminary report, we showed that this approach corrected the T-cell deficiency in two patients with X-linked severe combined immunodeficiency who were followed for 10 months after gene transfer.[7] We now report the effectiveness of the procedure in five patients with a follow-up of up to 30 months.

METHODS

Patients

Five consecutive patients without HLA-identical donors were enrolled in the trial between March 1999 and February 2000. The main characteristics of these boys at the time of diagnosis are shown in Table 1. The diagnosis of X-linked severe combined immunodeficiency was based on peripheral-blood lymphocyte counts and confirmed by γc mutation analysis. The protocol was approved by the French Drug Agency and the local ethics committee, and written informed consent was obtained from the parents, who were told that an alternative treatment (bone marrow transplantation) was available. All of the patients were kept in sterile isolation and received nonabsorbable antibiotics and intravenous immune globulin. Additional information about the five patients is available as Supplementary Appendix 1 with the full text of this article at http://www.nejm.org.

Retrovirus-Mediated Transduction

The vector containing the γc chain was derived from a defective Moloney murine leukemia virus and has been previously described.[7] With the patients under general anesthesia, 30 to 150 ml of bone marrow was obtained, and CD34+ cells in the marrow were selected for, as described below. These cells were stimulated to grow in X-vivo 10 medium (BioWhittaker, Walkersville, Md.) containing 4 percent fetal-calf serum (StemCell Technologies, Vancouver, B.C., Canada), 300 ng of stem-cell factor per milliliter (Amgen, Thousand Oaks, Calif.), 300 ng of Flt-3 ligand per milliliter (Immunex, Seattle), 60 ng of interleukin-3 per milliliter (Novartis, Rueil-Malmaison, France), and 100 ng of polyethylene glycol–conjugated megakaryocyte growth and differentiation factor per milliliter (Amgen). The cells were then transduced with a supernatant of the cultured γc-containing vector in the presence of the preceding cytokines and 4 ng of protamine sulfate per milliliter (Choay Sanofi, Gentilly, France). The procedure was carried out in sterile bags (Nexell Therapeutics, Irvine, Calif.) that were coated with 50 ng of human recombinant fibronectin per milliliter (Takara Shuzo, Shiga, Japan). The supernatant was replaced every 24 hours during the three-day transduction period. The number of cultured cells was increased by a factor of five to eight, and 14 million to 38 million CD34+ cells per kilogram of body weight were infused into the patients without preparative conditioning (Table 1).

Analysis of Immune Reconstitution

Immunofluorescence analysis, assays for proliferation of peripheral-blood mononuclear cells, analysis of the T-cell–receptor repertoire, and studies of natural-killer-cell cytotoxicity were performed as previously described.[7,9] The presence of serum antibodies against polioviruses, tetanus and diphtheria toxoids, *Haemophilus influenzae*, and *Streptococcus pneumoniae* was determined by enzyme linked immunosorbent assays. Levels of isohemagglutinins were measured by a hemagglutination assay. Antibody levels were determined one to three months after three immunizations had been administered. The interval between the last intravenous infusion of immune globulin and the determination of antibody levels was at least three months.

TABLE 1. CHARACTERISTICS OF THE PATIENTS.

PATIENT NO.	AGE AT TREATMENT	CLINICAL STATUS BEFORE TREATMENT	ENGRAFTMENT OF MATERNAL T CELLS	MUTATION	γc EXPRESSION BEFORE TREATMENT	INFUSED CELLS		CLINICAL STATUS AFTER TREATMENT	FOLLOW-UP
						CD34+	CD34γc+		
	mo		cells/mm³			cells/kg			yr
1	11	*Pneumocystis carinii* pneumonitis Protracted diarrhea Failure to thrive	0	Arg 289→stop	Yes	15 million	7 million–14 million	Well Normal growth	2.5
2	8	*Pneumocystis carinii* pneumonitis Protracted diarrhea Graft-versus-host disease–like lesions Failure to thrive	<10	Deletion of exon 6	No	16 million	5 million	Well Normal growth	2.3
3	10	Disseminated bacille Calmette–Guérin infection Adenovirus and respiratory syncytial virus infections in the lungs Protracted diarrhea Failure to thrive	0	Deletion of exon 4	No	14 million	5 million	Improving*	0.7
4	1	Well Free of infection	0	Tyr 219→stop	No	27 million	14 million	Well Normal growth	1.8
5	3	Graft-versus-host disease–like lesions	2000	Gln 285→Ala	No	38 million	20 million	Well Normal growth	1.6

*Eight months after gene therapy, Patient 3 underwent allogeneic stem cell transplantation.

Leukocyte Subgroups and Purification of CD34+ Cells

Peripheral-blood samples were separated into mononuclear cells and granulocytes by centrifugation and sorted by flow cytometry (FACS Vantage, Becton Dickinson Immunocytometry Systems, San Jose, Calif.). Isolation of CD34+ progenitor cells was performed by an immunomagnetic procedure (Miltenyi Biotec, Bergisch Gladbach, Germany). Two successive immunomagnetic procedures increased the purity of the CD34+ population to 99 percent.

Quantification of Transgene Integration

Genomic DNA was extracted from peripheral-blood mononuclear cells and amplified with use of quantitative polymerase chain reaction (PCR). Amplification, data acquisition, and analysis were performed with the use of a sequence detector (ABI PRISM 7700, Perkin Elmer, Norwalk, Conn.). Two sets of primers and probes were used in each PCR reaction. For the quantification of integrated transgene sequences, the primers positioned in the long terminal repeat and probe were as previously described.[10] The standard curve used as a reference for quantification of the viral copy number was based on serial dilutions of a plasmid ranging from 40 to 4 million copies. This plasmid contained two copies of the long terminal repeat and one of the human albumin sequence (Genethon III Laboratory, Evry, France).

To define the detection limit and linear range of duplex PCR, we used a standard curve consisting of a log-scale dilution of cells from an Epstein–Barr virus (EBV)–transformed B-cell line derived from a patient with X-linked severe combined immunodeficiency and containing approximately two copies of γc provirus per cell with uninfected cells from the same EBV-transformed B cell line. The lower limit of sensitivity of the method was 0.01 percent of γc-positive cells.

Quantification of T-Cell Antigen-Receptor Episomes

Analysis of T-cell antigen-receptor episomes in peripheral-blood mononuclear cells was performed by real-time quantitative PCR by means of the 5′ nuclease assay (TaqMan) with an ABI PRISM 7700 system (Perkin Elmer).[11,12] PCR conditions as well as primers and probe sequences are available on request.

Presence of Integrated Provirus after Long-Term Culture of CD34+ Cells

Purified CD34+ cells were cultured for six weeks on irradiated MS-5 stromal feeder layers in a limiting-dilution assay (10,000 to 150 cells per well) as described previously.[13] After six weeks, the cells were assayed for colony-forming units. Subsequently, for each dilution, all colony-forming units obtained on day 14 from the same dish were pooled. DNA was analyzed by PCR to determine the percentage of γc-positive dishes.

RESULTS

Clinical Outcome

After infusion of CD34+ cells that had been transduced in vitro with the *γc* gene, four of the five patients (Patients 1, 2, 4, and 5) had a clear-cut clinical improvement (Table 1). Pulmonary infections in Patient 1 and Patient 2 cleared and did not recur, and graft-versus-host–like skin lesions, a feature of severe combined immunodeficiency, disappeared in Patient 2 and Patient 5 within the first 50 days after gene therapy. Patient 1 and Patient 2 left the sterile environment on day 90, and Patient 4 and Patient 5 left on day 45. In Patient 1 and Patient 2, protracted diarrhea resolved, and parenteral nutrition was discontinued four months and three months after gene therapy, respectively. None of these four patients have subsequently had severe infections. Intravenous immune globulin was discontinued three to four months after gene therapy. Growth and psychomotor development have been normal to date. Patients 1, 2, 4, and 5 are now living at home in normal environmental conditions.

Patient 3, in whom reconstitution of T cells failed, underwent splenectomy four months after gene therapy for persistent splenomegaly caused by a disseminated bacille Calmette–Guérin infection. A rescue stem-cell transplantation from an unrelated donor matched at HLA-A, B, DR, and DQ loci but mismatched at one HLA-C locus was performed after eight months, according to the protocol. At the last follow-up visit, partial T-cell immunity had been restored in this patient.

T-Cell Development

In Patients 1, 2, and 4, the number of T cells increased progressively and reached normal values for age three to four months after gene therapy; they were within the normal range at the last follow-up visit (Fig. 1). In Patient 5, the initially high number of maternal T cells (Table 1) disappeared within three months after treatment, while autologous T cells appeared.

Quantitative analysis of provirus integration indicated that 100 percent of the T cells from Patients 1, 2, 4, and 5 contained the transgene (Fig. 2). On Southern blotting, there were one to three provirus integration sites per cell (data not shown). All T cells in Patients 2, 4, and 5 expressed cell-surface recep-

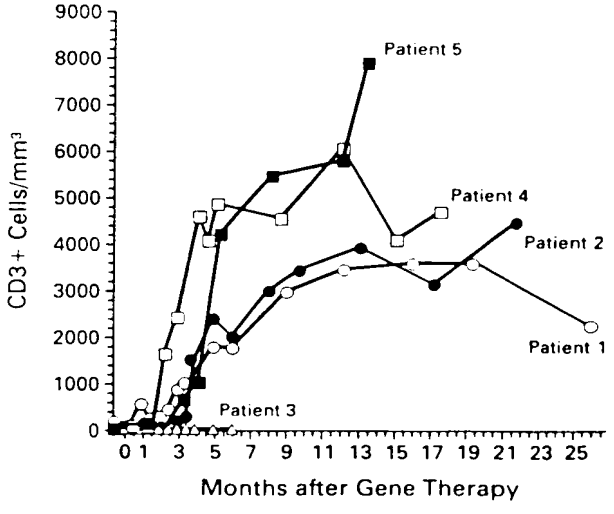

Figure 1. Absolute Numbers of CD3+ Cells after Gene Transfer in Patients 1 through 5.

tors with the γc chain. In all four patients, there was a normal distribution of T cells with α/β or γ/δ receptors, and the numbers of CD4+ and CD8+ T cells were similar to those in age-matched controls (data not shown). Conversely, no T cells were detected in the blood of Patient 3 up to six months after treatment (Fig. 1).

Analysis of naive (CD45RA+) and memory (CD45RO+) subgroups within CD4+ and CD8+ populations showed that most T cells had the phenotype of naive CD45RA+ T cells (Fig. 3A). We also assessed whether T cells were being synthesized by measuring the level of T-cell antigen-receptor episomes. Intrathymic rearrangements of genes encoding T-cell antigen receptors cause the formation of extrachromosomal DNA episomes, which mark T cells that have recently emigrated from the thymus to the periphery. As shown in Figure 3B, T-cell antigen-receptor episomes in Patients 1, 2, and 4 were first detected between day 60 and day 90, reached values found in age-matched controls, and remained stable for up to two years after gene transfer. Thirteen months after treatment, Patient 5 had 5500 CD45RA+ CD4+ T cells per cubic millimeter and 21,000 T-cell antigen-receptor episomes per 100,000 peripheral-blood mononuclear cells, respectively. These data correlated well with the development of a normal-sized thymus, as evaluated by ultrasonography (in Patients 1, 2, 4, and 5) and by magnetic resonance imaging in Patient 5 (respective size at one year or more, 23 by 15 by 11.5 mm, 21 by 13 by 10 mm, 27 by 34 by 13 mm, and 19 by 15 by 7 mm) (Fig. 3C).

Expression of 17 Vβ families of T-cell receptors in

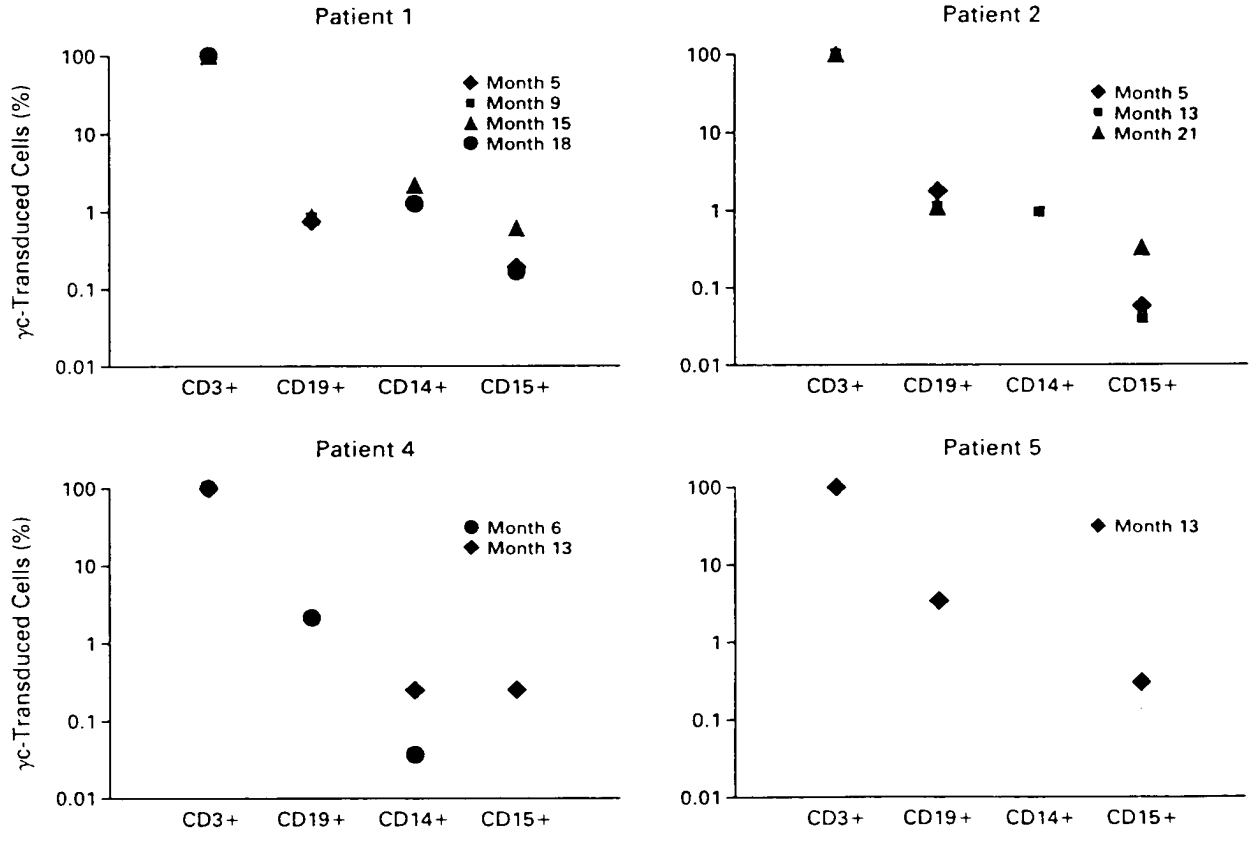

Figure 2. Frequency of Sorted T Cells (CD3+), B Cells (CD19+), Monocytes (CD14+), and Granulocytes (CD15+) Containing the Common γ (γc) Chain after Gene Therapy in Patients 1, 2, 4, and 5.

Real-time quantitative polymerase-chain-reaction analysis of DNA was used to determine the frequency of vector-containing cells, as described in the Methods section.

Patients 1, 2, 4, and 5 was similar to that in age-matched controls, and in these patients CD4+ and CD8+ T-cell populations remained stable. In all patients, a gaussian distribution of the lengths of complementarity-determining region 3 for 22 tested Vβ families of T-cell receptors was observed (see Supplementary Appendix 1).

Capacity for T-Cell Proliferation

At the last follow-up visit, T cells from Patients 1, 2, 4, and 5 exhibited normal proliferative responses to in vitro stimulation with phytohemagglutinin and anti-CD3 antibody (see Supplementary Appendix 1). Antigen-specific proliferative T-cell responses were also observed after immunization of those four patients with tetanus toxoid and polioviruses (see Supplementary Appendix 1). The addition of interleukin-2 to T cells from Patients 4 and 5 enhanced in vitro proliferative responses to tetanus toxoid. T cells from Patient 1, who was immunized with bacille Calmette–Guérin at two months of age, also had a proliferative response to tuberculin (purified protein derivative).

Development of Natural Killer Cells

Natural killer cells became detectable 15 to 45 days after gene therapy in Patients 2, 4, and 5 and 150 days after gene therapy in Patient 1 (Fig. 4). In Patients 2 and 4, and to a lesser magnitude in Patient 5, the levels of natural killer cells peaked two to four months after gene therapy and then gradually decreased. In Patient 3, natural killer cells were also detected in the blood beginning on day 45. These cells expressed γc as detected by immunofluorescence analysis (see Supplementary Appendix 1) and exhibited cytotoxic activity against K562 target cells (data not shown).

Serum Immunoglobulins and Antibody Production

Serum IgG, IgA, and IgM levels at 25, 21, and 13 months in Patients 1, 2, and 5, respectively, were

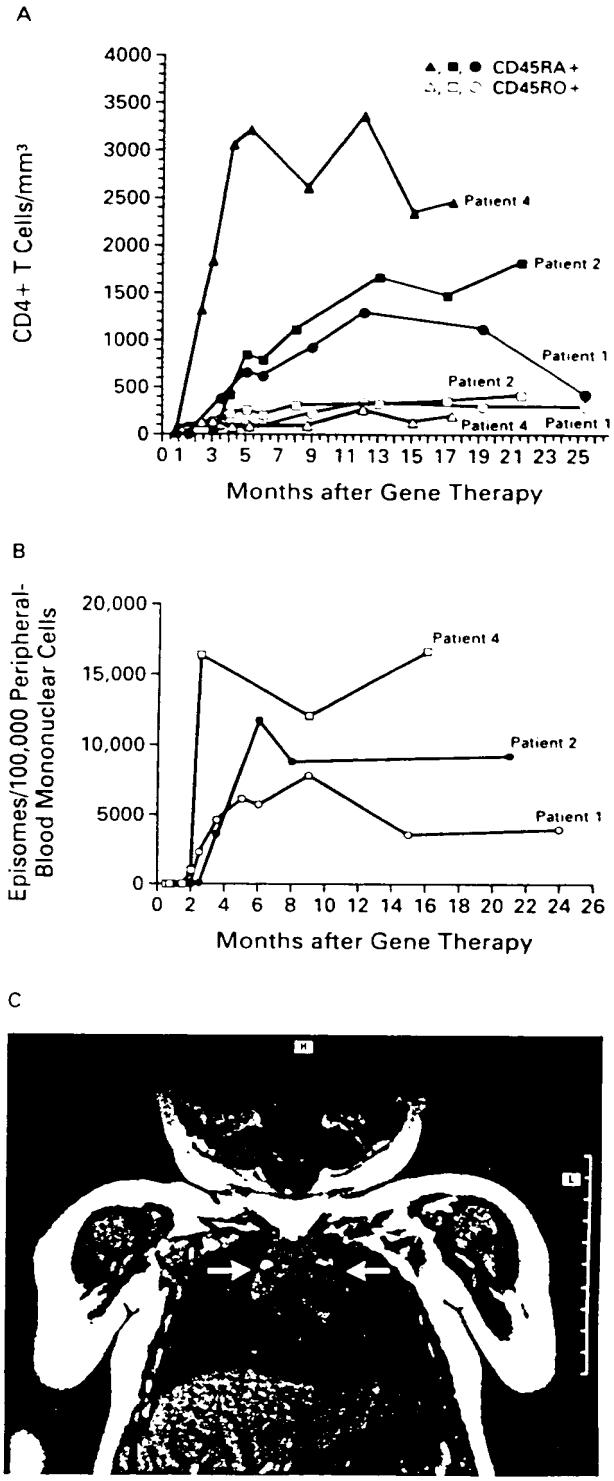

Figure 3. Numbers of Naive (CD45RA+) and Memory (CD45RO+) T Cells (Panel A) and Numbers of T-Cell Antigen-Receptor Episomes (Panel B) after Gene Therapy in Patients 1, 2, and 4 and Magnetic Resonance Image of a Coronal Section of the Thymus in Patient 5 Five Months after Gene Therapy (Panel C).

In Panel A, phenotypic quantification of naive and memory CD4+ T cells was performed with the use of double staining with fluorochrome-conjugated antibodies against CD4 and CD45RA or CD45RO. In Panel B, numbers of T-cell antigen-receptor episomes in peripheral-blood mononuclear cells were evaluated at different times. The normal range of T-cell antigen-receptor episomes for age-matched controls is 2500 to 20,000 per 100,000 peripheral-blood mononuclear cells. Arrows in Panel C show a normal-sized thymus after reconstitution of T cells.

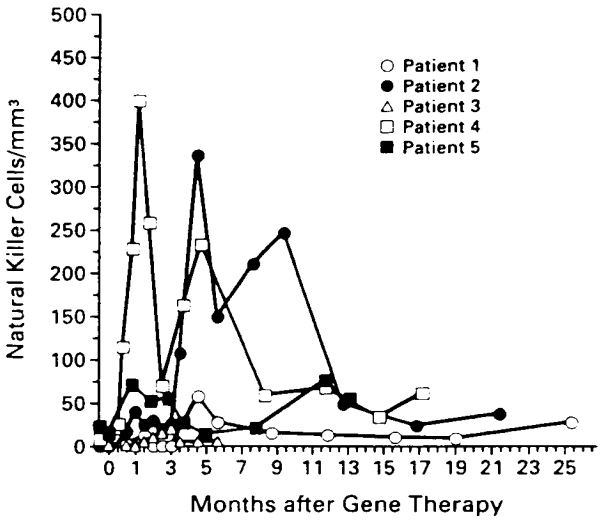

Figure 4. Absolute Numbers of CD56+ and CD16+ Cells per Cubic Millimeter of Whole Blood after Gene Therapy in Patients 1 through 5.

within the age-related normal range (Fig. 5). Low IgG and IgA levels persisted in Patient 4 (Fig. 5). Antibodies against tetanus toxoid, diphtheria toxoid, and poliovirus antigens were first found one month after the third immunization (Table 2) and persisted for more than six months in Patients 1, 2, and 4. Antibodies against *S. pneumoniae* in Patient 2 and *H. influenzae* in Patient 1 and Patient 2 were also detected. In contrast, immunization of Patient 5 failed to elicit an antibody response. Isohemagglutinins were consistently detected in the serum of Patients 1, 2, and 4 one year or more after gene therapy (Table 2). In three patients, the percentage of CD27+ and CD19+ B cells was similar to that of age-matched controls (see Supplementary Appendix 1).

Integration and Expression of γc Provirus

In Patients 1, 2, 4, and 5, all CD3+ T cells carried the γc transgene, as compared with 1 to 5 percent of B cells, 0.05 to 2 percent of monocytes, and 0.05 to 0.5 percent of granulocytes (Fig. 2). The frequency of γc-containing T cells, B cells, monocytes, and granulocytes was stable during the study period (Fig. 2). In Patients 2, 4, and 5, the presence of the γc gene coincided with the expression of γc chains (see Supplementary Appendix 1). In bone marrow samples obtained from Patient 2 and Patient 4 21 and 13 months, respectively, after gene transfer, 1 to 5 percent of colony-forming units derived from cultured CD34+ cells contained the transgene (frequency of long-term-culture initiating cells, 1:1000 in Patient 2 and 1:500 in Patient 4) (data not shown).

Patient 3

Reconstitution of T cells failed to occur in Patient 3 (Fig. 1), despite the presence of γc-positive cells, as detected by PCR and immunofluorescence analysis of peripheral-blood mononuclear cells from day 30 up to four months after gene transfer. After splenectomy, a strong γc signal was detected among sorted CD19+ and CD16+ cells by nonquantitative PCR analysis. There were no CD3+ T cells in the spleen, and provirus (i.e., vector) was not detected in a bone marrow sample obtained at the time of splenectomy.

DISCUSSION

We found that four of five patients with X-linked severe combined immunodeficiency due to a deficiency of the γc chain who were treated with autologous CD34+ cells from bone marrow that had been transduced ex vivo with the γc gene showed evidence of a functional immune system and sustained clinical benefit. These results extend a preliminary report of two patients treated in this way.[7] The gene-therapy protocol we used is safe, and no evidence of the emergence of a replication-competent retrovirus has been detected.

The evidence that virtually all T cells and natural killer cells but fewer B cells and myeloid cells were transduced suggests that γc expression gives progenitors of T cells and natural killer cells a selective growth advantage. Since transduced monocytes, granulocytes, and colonies derived from long-term cultures of transduced CD34+ cells were consistently detected one to two years after gene transfer, it is likely that long-lived immature progenitor cells were targeted by the vector. Moreover, the persistence of T-cell antigen-receptor episomes,[11,12] naive T cells, and the development of a normal-sized thymus indicate ongoing formation of T cells and thymopoiesis, which most likely originated from transduced CD34+ progenitors. These findings suggest that both committed myeloid and lymphoid progenitor cells were transduced (implying that these cells persist in the bone marrow for at least one to two years) or that uncommitted pluripotent progenitor cells were transduced by the γc-containing vector. Evaluation of provirus integration sites in myeloid and lymphoid cells[14,15] should help clarify this issue.

In our four successfully treated patients, the pattern of restoration of T cells differed from that observed after transplantation of haploidentical hematopoietic stem cells in patients with severe combined immunodeficiency.[3,4] After the latter, T cells usually begin to appear within four to six months, and the

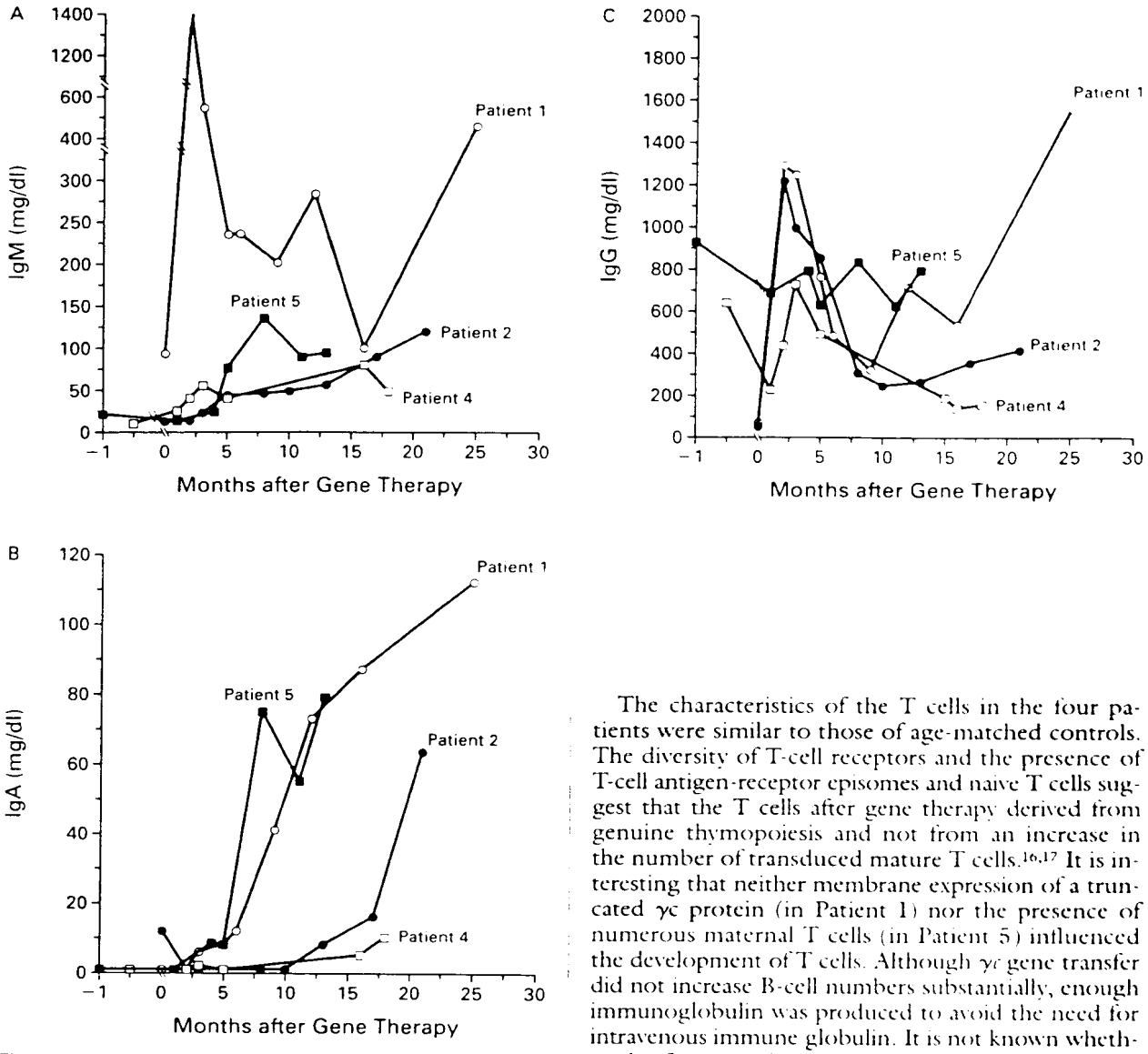

Figure 5. Serum Levels of IgM (Panel A), IgA (Panel B), and IgG (Panel C) after Gene Therapy in Patients 1, 2, 4, and 5.

In Patient 1, the peak level of monoclonal IgM occurred two months after gene therapy.

number of T cells in peripheral blood rarely exceeds 2000 per cubic millimeter.[3,4] In contrast, after gene therapy, T cells appeared within two to four months, at levels of 2000 to 8000 per cubic millimeter. The absence of graft-versus-host disease and the ex vivo activation of CD34+ cells with cytokines could have contributed to the rapid reconstitution.

The characteristics of the T cells in the four patients were similar to those of age-matched controls. The diversity of T-cell receptors and the presence of T-cell antigen-receptor episomes and naive T cells suggest that the T cells after gene therapy derived from genuine thymopoiesis and not from an increase in the number of transduced mature T cells.[16,17] It is interesting that neither membrane expression of a truncated γc protein (in Patient 1) nor the presence of numerous maternal T cells (in Patient 5) influenced the development of T cells. Although γc gene transfer did not increase B-cell numbers substantially, enough immunoglobulin was produced to avoid the need for intravenous immune globulin. It is not known whether the few transduced B cells account for the production of antibodies in these patients or whether nontransduced B cells are also involved.[18] Since there were more detectable memory B cells (CD27+ and CD19+) than transduced B cells, it is possible that γc-negative B cells retain some function.

In conclusion, our study demonstrates that the infusion of autologous γc-transduced cells, despite the low efficiency of the transduction process, can repair the immune system in patients with X-linked severe combined immunodeficiency. Although the repair is incomplete, it is sufficient to provide protective immunity. Despite an obvious requirement for long-term assessment and further analysis in a larger cohort of patients, these results suggest that a similar approach

TABLE 2. PEAK ANTIBODY RESPONSES AFTER IMMUNIZATION.*

ANTIBODY ASSAY	PATIENT 1	PATIENT 2	PATIENT 4	PATIENT 5	CONTROLS
Diphtheria toxoid (IU/ml)	3	93	22	<0.1	>0.10
Tetanus toxoid (IU/ml)	3	63	89	<0.1	>0.10
Poliovirus titer					
First	1:640	1:640	1:20	0	>1:40
Second	1:320	1:640	1:80	1:20	>1:40
Third	1:160	1:160	1:40	0	>1:0
Anti-A antibody titer	1:64	1:32	1:8	1:4	>1:8
Anti-B antibody titer	1:32	—	—	—	—
Haemophilus influenzae (%)†	26	16	ND	ND	>10
Streptococcus pneumoniae (μg/ml)	ND	8	ND	ND	>0.3

*Patients were immunized three times with diphtheria toxoid, tetanus toxoid, and poliovirus between month 4 and month 6, they were immunized with *Streptococcus pneumoniae* and *Haemophilus influenzae* one year after gene therapy. Serum antibodies were measured in serum samples drawn every three months thereafter. ND denotes not done.

†A positive value is more than 10 percent.

could be used for other forms of severe combined immunodeficiency.[19-24]

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On the Edge of the Sahara

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